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(54) Title: MODULATION OF IMMUNOSTIMULATORY ACTIVITY OF IMMUNOSTIMULATORY OLIGONUCLEOTIDE ANALOGS BY POSITIONAL CHEMICAL CHANGES

(57) Abstract: The invention relates to the therapeutic use of oligonucleotides or oligonucleotide analogs as immunostimulatory agents in immunotherapy applications. The invention provides methods for enhancing the immune response caused by immunostimulatory oligonucleotide compounds.

**MODULATION OF IMMUNOSTIMULATORY ACTIVITY OF
IMMUNOSTIMULATORY OLIGONUCLEOTIDE ANALOGS BY
POSITIONAL CHEMICAL CHANGES**

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BACKGROUND OF THE INVENTION

Field of the invention

The invention relates to the therapeutic use of oligonucleotides or
oligonucleotide analogs as immunostimulatory agents in immunotherapy
10 applications.

Summary of the related art

Oligonucleotides have become indispensable tools in modern molecular
biology, being used in a wide variety of techniques, ranging from diagnostic
15 probing methods to PCR to antisense inhibition of gene expression and
immunotherapy applications. This widespread use of oligonucleotides has led to
an increasing demand for rapid, inexpensive and efficient methods for
synthesizing oligonucleotides.

The synthesis of oligonucleotides for antisense and diagnostic applications
20 can now be routinely accomplished. See e.g., *Methods in Molecular Biology, Vol 20:
Protocols for Oligonucleotides and Analogs* pp. 165-189 (S. Agrawal, Ed., Humana
Press, 1993); *Oligonucleotides and Analogues: A Practical Approach*, pp. 87-108 (F.
Eckstein, Ed., 1991); and Uhlmann and Peyman, *supra*. Agrawal and Iyer, *Curr.
Op. in Biotech.* 6: 12 (1995); and *Antisense Research and Applications* (Crooke and
25 Lebleu, Eds., CRC Press, Boca Raton, 1993). Early synthetic approaches included
phosphodiester and phosphotriester chemistries. Khorana et al., *J. Molec. Biol.* 72:
209 (1972) discloses phosphodiester chemistry for oligonucleotide synthesis.
Reese, *Tetrahedron Lett.* 34: 3143-3179 (1978), discloses phosphotriester chemistry
for synthesis of oligonucleotides and polynucleotides. These early approaches
30 have largely given way to the more efficient phosphoramidite and

H-phosphonate approaches to synthesis. Beaucage and Caruthers, *Tetrahedron Lett.* 22: 1859-1862 (1981), discloses the use of deoxynucleoside phosphoramidites in polynucleotide synthesis. Agrawal and Zamecnik, U.S. Patent No. 5,149,798 (1992), discloses optimized synthesis of oligonucleotides by the H-phosphonate approach.

Both of these modern approaches have been used to synthesize oligonucleotides having a variety of modified internucleotide linkages. Agrawal and Goodchild, *Tetrahedron Lett.* 28: 3539-3542 (1987), teaches synthesis of oligonucleotide methylphosphonates using phosphoramidite chemistry.

Connolly et al., *Biochemistry* 23: 3443 (1984), discloses synthesis of oligonucleotide phosphorothioates using phosphoramidite chemistry. Jager et al., *Biochemistry* 27: 7237 (1988), discloses synthesis of oligonucleotide phosphoramidates using phosphoramidite chemistry. Agrawal et al., *Proc. Natl. Acad. Sci. USA* 85: 7079-7083 (1988), discloses synthesis of oligonucleotide phosphoramidates and phosphorothioates using H-phosphonate chemistry.

More recently, several researchers have demonstrated the validity of the use of oligonucleotides as immunostimulatory agents in immunotherapy applications. The observation that phosphodiester and phosphorothioate oligonucleotides can induce immune stimulation has created interest in developing this side effect as a therapeutic tool. These efforts have focused on phosphorothioate oligonucleotides containing the dinucleotide CpG.

Kuramoto et al., *Jpn. J. Cancer Res.* 83: 1128-1131 (1992) teaches that phosphodiester oligonucleotides containing a palindrome that includes a CpG dinucleotide can induce interferon-alpha and gamma synthesis and enhance natural killer activity. Krieg et al., *Nature* 371: 546-549 (1995) discloses that phosphorothioate CpG-containing oligonucleotides are immunostimulatory. Liang et al., *J. Clin. Invest.* 98: 1119-1129 (1996) discloses that such oligonucleotides activate human B cells.

Pisetsky, D. S.; Rich C. F., *Life Sci.* 54: 101 (1994), teaches that the immunostimulatory activity of CpG-oligos is further enhanced by the presence of

phosphorothioate (PS) backbone on these oligos. Tokunaga, T.; Yamamoto, T.; Yamamoto, S. *Jap. J. Infect. Dis.* 52: 1 (1999), teaches that immunostimulatory activity of CpG-oligos is dependent on the position of CpG-motif and the sequences flanking CpG-motif. The mechanism of activation of immune stimulation by CpG-oligos has not been well understood. Yamamoto, T.; Yamamoto, S.; Kataoka, T.; Tokunaga, T., *Microbiol. Immunol.* 38: 831 (1994), however, suggests that CpG-oligos trigger immune cascade by binding to an intracellular receptor/protein, which is not characterized yet.

Several researchers have found that this ultimately triggers stress kinase pathways, activation of NF- κ B and induction of various cytokines such as IL-6, IL-12, γ -IFN, and TNF- α . (See *e.g.*, Klinman, D. M.; Yi, A. K.; Beaucage, S. L.; Conover, J.; Krieg, A. M., *Proc. Natl. Acad. Sci. U. S. A.* 93: 2879 (1996); Sparwasser, T.; Miethke, T.; Lipford, G. B.; Erdmann, A.; Haecker, H.; Heeg, K.; Wagner, H., *Eur. J. Immunol.* 27: 1671 (1997); Lipford, G. B.; Sparwasser, T.; Bauer, M.; Zimmermann, S.; Koch, E. S.; Heeg, K.; Wagner, H. *Eur. J., Immunol.* 27: 3420 (1997); Sparwasser, T.; Koch, E. S.; Vabulas, R. M.; Lipford, G. B.; Heeg, K.; Ellart, J. W.; Wagner, H., *Eur. J. Immunol.* 28: 2045 (1998); and Zhao, Q.; Temsamani, J.; Zhou, R. Z.; Agrawal, S. *Antisense Nucleic Acid Drug Dev.* 7: 495 (1997).)

The use of CpG-PS-oligos as antitumor, antiviral, antibacterial and antiinflammatory agents and as adjuvants in immunotherapy has been reported. (See *e.g.*, Dunford, P. J.; Mulqueen, M. J.; Agrawal, S. *Antisense 97: Targeting the Molecular Basis of Disease*, (Nature Biotechnology) Conference abstract, 1997, pp 40; Agrawal, S.; Kandimalla E. R. *Mol. Med. Today* 6: 72 (2000); Chu. R. S.; Targoni, O. S.; Krieg, A. M.; Lehmann, P. V.; Harding, C. V. *J. Exp. Med.* 186: 1623 (1997); Zimmermann, S.; Egeter, O.; Hausmann, S.; Lipford, G. B.; Rocken, M.; Wagner, H.; Heeg, K. *J. Immunol.* 160: 3627 (1998).) Moldoveanu *et al.*, *Vaccine* 16: 1216-124 (1998) teaches that CpG-containing phosphorothioate oligonucleotides enhance immune response against influenza virus. McCluskie and Davis, *J. Immunol.* 161: 4463-4466 (1998) teaches that CpG-containing oligonucleotides act as potent adjuvants, enhancing immune response against hepatitis B surface antigen.

Zhao, Q.; Temsamani, J.; Idarola, P.; Jiang, Z.; Agrawal, S. *Biochem. Pharmacol.* 51: 173 (1996), teaches that replacement of deoxynucleosides in a CpG-motif with 2'-O-methylribonucleosides suppresses immunostimulatory activity, suggesting that a rigid C3'-endo conformation induced by 2'-O-methyl
5 modification does not allow proper recognition and/or interaction of CpG-motif with the proteins involved in the immunostimulatory pathway. This reference further teaches that substitution of a methyl group for an unbridged oxygen on the phosphate group between C and G of a CpG-motif suppresses immune stimulatory activity, suggesting that negative charge on phosphate group is
10 essential for protein recognition and interaction.

Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* 9: 3453 (1999), teaches that substitution of one or two 2'-deoxynucleosides adjacent to CpG-motif with 2'- or 3'-O-methylribonucleosides on the 5'-side causes a decrease in immunostimulatory activity, while the same substitutions have insignificant effect
15 when they were placed on the 3'-side of the CpG-motif. However, Zhao, Q.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* 10: 1051 (2000), teaches that the substitution of a deoxynucleoside two or three nucleosides away from the CpG-motif on the 5'-side with one or two 2'-O-methoxyethyl- or 2'- or 3'-O-methylribonucleosides results in a significant increase in immunostimulatory
20 activity.

The precise structural requirements and specific functional groups of CpG-motif necessary for the recognition of protein/receptor factor that is responsible for immune stimulation have not yet been studied in detail. There is, therefore, a need for new immunostimulatory motifs which may provide improved
25 immunostimulatory activity.

BRIEF SUMMARY OF THE INVENTION

The invention provides methods for enhancing the immune response caused by immunostimulatory oligonucleotide compounds. The methods according to the invention enable increasing the immunostimulatory effect for immunotherapy applications. Thus, the invention further provides methods for making and using such oligonucleotide compounds.

The present inventors have surprisingly discovered that positional modification of immunostimulatory oligonucleotides dramatically affects their immunostimulatory capabilities. In particular, modifications in the immunostimulatory domain and/or the potentiation domain enhance the immunostimulatory effect in a reproducible and predictable manner.

In a first aspect, the invention provides immunostimulatory oligonucleotide compounds comprising an immunostimulatory domain and, optionally, one or more potentiation domains. In some embodiments, the immunostimulatory domain comprises a dinucleotide analog that includes a non-naturally occurring pyrimidine base. In some embodiments, the immunostimulatory domain and/or the potentiation domain include an immunostimulatory moiety at a specified position, as described hereinbelow. In some embodiments, the immunostimulatory oligonucleotide comprises a 3'-3' linkage. In one embodiment, such 3'-3' linked oligonucleotides have two accessible 5'-ends.

In a second aspect, the invention provides methods for modulating the immunostimulatory effect of an immunostimulatory oligonucleotide compound. In some embodiments, the method comprises introducing into the immunostimulatory domain a dinucleotide analog that includes a non-naturally occurring pyrimidine base. In some embodiments, the method comprises introducing into the immunostimulatory domain and/or potentiation domain an immunostimulatory moiety at a specified position, as described hereinbelow. In some embodiments, the method comprises introducing into the oligonucleotide a 3'-3' linkage.

In a third aspect, the invention provides methods for generating an immune response in a patient, such methods comprising administering to the patient an immunostimulatory oligonucleotide compound according to the invention.

5 In a fourth aspect, the invention provides methods for therapeutically treating a patient having disease caused by a pathogen, such methods comprising administering to the patient an immunostimulatory oligonucleotide compound according to the invention.

10 In a fifth aspect, the invention provides methods for treating a cancer patient, such methods comprising administering to the patient an immunostimulatory oligonucleotide compound according to the invention.

15 In a sixth aspect, the invention provides methods for treating autoimmune disorders, such as autoimmune asthma, such methods comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to the invention. Administration is carried out as described for the third aspect of the invention.

20 In a seventh aspect, the invention provides methods for treating airway inflammation or allergies, such methods comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to the invention. Administration is carried out as described for the third aspect of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows results of proliferation assays using oligonucleotides having 1',2'-dideoxyribose substitutions at various positions.

5 Figure 2 shows results of spleen weight assays using oligonucleotides having 1',2'-dideoxyribose substitutions at various positions.

Figure 3 shows results of proliferation assays using different oligonucleotides having 1',2'-dideoxyribose substitutions at various positions.

Figure 4 shows results of spleen weight assays using different oligonucleotides having 1',2'-dideoxyribose substitutions at various positions.

10 Figure 5 shows results of proliferation assays using oligonucleotides having C3-linker substitutions at various positions.

Figure 6 shows results of spleen weight assays using oligonucleotides having C3-linker substitutions at various positions.

15 Figure 7 shows results of proliferation assays using oligonucleotides having Spacer 9 or Spacer 18 substitutions at various positions.

Figure 8 shows results of spleen weight assays using oligonucleotides having Spacer 9 or Spacer 18 substitutions at various positions.

Figure 9 shows results of proliferation assays using oligonucleotides having amino-linker substitutions at various positions.

20 Figure 10 shows results of spleen weight assays using oligonucleotides having amino-linker substitutions at various positions.

Figure 11 shows results of proliferation assays using oligonucleotides having 3'-deoxynucleoside substitutions at various positions.

25 Figure 12 shows results of spleen weight assays using oligonucleotides having 3'-deoxynucleoside substitutions at various positions.

Figure 13 shows results of proliferation assays using oligonucleotides having methylphosphonate substitutions at various positions.

Figure 14 shows results of spleen weight assays using oligonucleotides having methylphosphonate substitutions at various positions.

Figure 15 shows results of proliferation assays using oligonucleotides having 2'-O-methylribonucleoside or 2'-O-methoxyethyl substitutions at various
5 positions.

Figure 16 shows results of spleen weight assays using oligonucleotides having 2'-O-methylribonucleoside or 2'-O-methoxyethyl substitutions at various positions.

Figure 17 shows results of proliferation assays using oligonucleotides
10 having 5'-3', 5'-5', or 3'-3' linkage substitutions at various positions.

Figure 18 shows results of spleen weight assays using oligonucleotides having β -L-deoxynucleotide substitutions at various positions.

Figure 19 shows results of spleen weight assays using oligonucleotides having 2'-O-propargyl substitutions at various positions.

Figure 20 shows results of spleen weight assays using oligonucleotides
15 having various substitutions at various positions.

Figure 21 shows results of spleen weight assays using oligonucleotides having 7-deazaguanine substitution within the immunostimulatory dinucleotide.

Figure 22 shows results of proliferation assays using oligonucleotides
20 having 6-thioguanine substitution within the immunostimulatory dinucleotide.

Figure 23 shows results of spleen weight assays using oligonucleotides having 5-hydroxycytosine or N4-ethylcytosine substitution within the immunostimulatory dinucleotide.

Figure 24 shows results of spleen weight assays using oligonucleotides
25 having 5-hydroxycytosine or N4-ethylcytosine substitution within the immunostimulatory dinucleotide.

Figure 25 shows results of proliferation assays using oligonucleotides having arabinofuranosylcytosine (aracytidine; Ara-C) substitution within the immunostimulatory dinucleotide.

5 Figure 26 shows results of spleen weight assays using oligonucleotides having 4-thiouracil substitution within the immunostimulatory dinucleotide.

Figure 27 shows the chemical structure of a CpG-motif, showing functional groups on cytosine that serve as hydrogen bond acceptor and hydrogen bond donor groups.

10 Figure 28 shows the chemical structures of cytosine (1) and cytosine analogs (2-7). In the nucleosides cytidine, deoxycytidine, and related analogs, the substituent R is ribose or 2'-deoxyribose.

DETAILED DESCRIPTION

The invention relates to the therapeutic use of oligonucleotides and oligonucleotide analogs as immunostimulatory agents for immunotherapy applications. The patents and publications cited herein reflect the level of
5 knowledge in the field and are hereby incorporated by reference in their entirety. In the event of conflict between any teaching of any reference cited herein and the present specification, the latter shall prevail, for purposes of the invention.

The invention provides methods for enhancing the immune response caused by immunostimulatory oligonucleotide compounds for immunotherapy
10 applications. Thus, the invention further provides compounds having optimal levels of immunostimulatory effect for immunotherapy and methods for making and using such oligonucleotide compounds.

The present inventors have surprisingly discovered that positional chemical modifications introduced in immunostimulatory oligonucleotides
15 dramatically affect their immunostimulatory capabilities. In particular, modifications in the immunostimulatory domain and/or the potentiation domain can enhance the immunostimulatory effect in a reproducible manner for desired applications.

In a first aspect, the invention provides immunostimulatory
20 oligonucleotide compounds comprising an immunostimulatory domain and, optionally, one or more potentiation domains. In certain preferred embodiments, the immunostimulatory domain comprises a dinucleotide analog that includes a non-natural pyrimidine nucleoside.

For purposes of all aspects of the invention, the term "oligonucleotide"
25 includes polymers of two or more deoxyribonucleosides, or any modified nucleoside, including 2'- or 3'-substituted nucleosides, 2'- or 3'-O-substituted ribonucleosides, deazanucleosides, or any combination thereof. Such monomers may be coupled to each other by any of the numerous known internucleoside linkages. In certain preferred embodiments, these internucleoside linkages may

be phosphodiester, phosphotriester, phosphorothioate, phosphorodithioate, or phosphoramidate linkages, including 3'-5', 2'-5', 3'-3', and 5'-5' linkages of any of the foregoing, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or
5 having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines and adamantane. The term oligonucleotide also encompasses peptide nucleic acids (PNA), peptide nucleic acids with phosphate groups (PHONA), locked nucleic acids (LNA), morpholinonucleic acids, and oligonucleotides comprising non-pentose sugar (e.g. hexose) or abasic sugar
10 backbones or backbone sections, as well as oligonucleotides that include backbone sections with non-sugar linker or spacer groups, as further described hereinbelow.

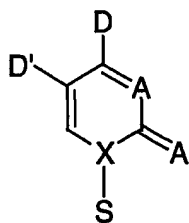
For purposes of the invention the terms "2'-substituted" and "3'-substituted" mean (respectively) substitution of the 2' (or 3') position of the pentose moiety with a halogen (preferably Cl, Br, or F), or an -O-lower alkyl
15 group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxy, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside) or an
20 amino group, but not with a 2' (or 3') H group.

For purposes of the invention, the term "immunostimulatory oligonucleotide compound" means a compound comprising an immunostimulatory dinucleotide, without which the compound would not have an immunostimulatory effect. An "immunostimulatory dinucleotide" is a
25 dinucleotide having the formula 5'-pyrimidine-purine-3', wherein "pyrimidine" is a natural or non-natural pyrimidine nucleoside and "purine" is a natural or non-natural purine nucleoside. One such immunostimulatory dinucleotide is CpG. The terms "CpG" and "CpG dinucleotide" refer to the dinucleotide 5'-deoxycytidine-deoxyguanosine-3', wherein p is an internucleotide linkage,
30 preferably selected from the group consisting of phosphodiester, phosphorothioate, and phosphorodithioate.

For purposes of the invention, a "dinucleotide analog" is an immunostimulatory dinucleotide as described above, wherein either or both of the pyrimidine and purine nucleosides is a non-natural nucleoside. A "non-natural" nucleoside is one that includes a non-naturally occurring base and/or a non-naturally occurring sugar moiety. For purposes of the invention, a base is considered to be non-natural if it is not selected from the group consisting of thymine, guanine, cytosine, adenine, and uracil. The terms "C*pG" and "CpG*" refer to immunostimulatory dinucleotide analogs comprising a cytidine analog (non-natural pyrimidine nucleoside) or a guanosine analog (non-natural purine nucleoside), respectively.

Figure 27 shows the chemical structure of a CpG-motif, showing the functional groups on cytosine that serve as hydrogen bond acceptor and hydrogen bond donor groups. Cytosine has two hydrogen bond acceptor groups at positions 2 (keto-oxygen) and 3 (nitrogen), and a hydrogen bond donor group at the 4-position (amino group). These groups can serve as potential recognizing and interacting groups with receptors that are responsible for immune stimulation. Figure 28 shows cytosine analogs that are isostructural with natural cytosine, including 5-methyl-deoxycytosine (2), 5-methyl-deoxyisocytosine (3), 5-hydroxy-deoxycytosine (4), deoxyuridine (5), N4-ethyl-deoxycytosine (6), and deoxy-P-base (7).

In one embodiment, therefore, the immunostimulatory dinucleotide comprises a pyrimidine nucleoside of structure (I):



(I)

wherein D is a hydrogen bond donor, D' is selected from the group consisting of hydrogen, hydrogen bond donor, hydrogen bond acceptor, hydrophilic group,

hydrophobic group, electron withdrawing group and electron donating group, A is a hydrogen bond acceptor, X is carbon or nitrogen, and S is a pentose or hexose sugar ring linked to the pyrimidine base. In some embodiments, the pyrimidine nucleoside is a non-natural pyrimidine nucleoside, i.e., the compound of structure (I) is not cytidine or deoxycytidine.

In some embodiments, the base moiety in (I) is a non-naturally occurring pyrimidine base. Examples of preferred non-naturally occurring pyrimidine bases include, without limitation, 5-hydroxycytosine, 5-hydroxymethylcytosine, N4-alkylcytosine, preferably N4-ethylcytosine, and 4-thiouracil. In some embodiments, the sugar moiety S in (I) is a non-naturally occurring sugar moiety. For purposes of the present invention, a "naturally occurring sugar moiety" is ribose or 2'-deoxyribose, and a "non-naturally occurring sugar moiety" is any sugar other than ribose or 2'-deoxyribose that can be used in the backbone for an oligonucleotide.. Arabinose and arabinose derivatives are examples of a preferred non-naturally occurring sugar moieties.

Immunostimulatory domains according to the invention may include immunostimulatory moieties on one or both sides of the immunostimulatory natural dinucleotide or non-natural dinucleotide analog. For example, an immunostimulatory domain could be depicted as

5'-----X1-X2-Y-Z-X3-X4-----3'

wherein Y represents cytidine or a non-natural pyrimidine nucleoside analog, Z represents guanosine or a non-natural purine nucleoside analog, and each X independently represents a nucleoside or an immunostimulatory moiety according to the invention. An "immunostimulatory moiety" is a chemical structure at a particular position within the immunostimulatory domain or the potentiation domain that causes the immunostimulatory oligonucleotide to be more immunostimulatory than it would be in the absence of the immunostimulatory moiety.

Preferred immunostimulatory moieties include modifications in the phosphate backbones including without limitation methylphosphonates, methylphosphonothioates phosphotriesters, phosphothiotriesters phosphorothioates, phosphorodithioates, triester prodrugs, sulfones, sulfonamides, sulfamates, formacetal, N-methylhydroxylamine, carbonate, carbamate, boranophosphonate, phosphoramidates, especially primary aminophosphoramidates, N3 phosphoramidates and N5 phosphoramidates, and stereospecific linkages (e.g., (R)- or (S)-phosphorothioate, alkylphosphonate, or phosphotriester linkages). Preferred immunostimulatory moieties according to the invention further include nucleosides having sugar modifications, including without limitation 2'-substituted pentose sugars including without limitation 2'-O-methylribose, 2'-O-methoxyethylribose, 2'-O-propargylribose, and 2'-deoxy-2'-fluororibose; 3'-substituted pentose sugars, including without limitation 3'-O-methylribose; 1',2'-dideoxyribose; hexose sugars, including without limitation arabinose, 1'-methylarabinose, 3'-hydroxymethylarabinose, 4'-hydroxymethylarabinose, and 2'-substituted arabinose sugars; and alpha-anomers.

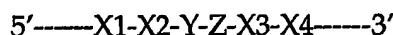
Preferred immunostimulatory moieties according to the invention further include oligonucleotides having other carbohydrate backbone modifications and replacements, including peptide nucleic acids (PNA), peptide nucleic acids with phosphate groups (PHONA), locked nucleic acids (LNA), morpholinonucleic acids, and oligonucleotides having backbone sections with alkyl linkers or amino linkers. The alkyl linker may be branched or unbranched, substituted or unsubstituted, and chirally pure or a racemic mixture. Most preferably, such alkyl linkers have from about 2 to about 18 carbon atoms. In some preferred embodiments such alkyl linkers have from about 3 to about 9 carbon atoms. Such alkyl linkers include polyethyleneglycol linkers $[-O-CH_2-CH_2-]_n$ ($n = 2-9$). In some preferred embodiments, such alkyl linkers may include peptides or amino acids.

Preferred immunostimulatory moieties according to the invention further include DNA isoforms, including without limitation β -L-deoxynucleosides and

alpha-deoxynucleosides. Preferred immunostimulatory moieties according to the invention further include nucleosides having unnatural internucleoside linkage positions, including without limitation 2'-5', 2'-2', 3'-3' and 5'-5' linkages.

Preferred immunostimulatory moieties according to the invention further
 5 include nucleosides having modified heterocyclic bases, including without limitation 5-hydroxydeoxycytidine, 5-hydroxymethyldeoxycytidine, N4-alkyldeoxycytidine, preferably N4-ethyldeoxycytidine, 4-thiouridine, 6-thiodeoxyguanosine, 7-deazaguanosine, and deoxyribonucleosides of nitropyrrole, C5-propynylpyrimidine, and diaminopurine, including without
 10 limitation 2,6-diaminopurine.

By way of specific illustration and not by way of limitation, for example, in the immunostimulatory domain described earlier



a nucleoside methylphosphonate at position X3 or X4 is an immunostimulatory
 15 moiety, a substituted or unsubstituted alkyl linker at position X1 is an immunostimulatory moiety, and a β -L-deoxynucleoside at position X1 is an immunostimulatory moiety. See Table 1 below for representative positions and structures of immunostimulatory moieties within the immunostimulatory domain.

20

Table 1

Position	TYPICAL IMMUNOSTIMULATORY MOIETIES
X1	C3-alkyl linker, 2-aminobutyl-1,3-propanediol linker (amino linker), β -L-deoxynucleoside
X2	2-aminobutyl-1,3-propanediol linker
X3	nucleoside methylphosphonate
X4	nucleoside methylphosphonate, 2'-O-methyl-ribonucleoside

In some embodiments, the immunostimulatory oligonucleotide further comprises a potentiation domain

A "potentiation domain" is a region of an immunostimulatory oligonucleotide analog, other than the immunostimulatory domain, that causes the oligonucleotide to be more immunostimulatory if it contains the potentiation domain than the oligonucleotide would be in the absence of the potentiation domain. The potentiation domain can be upstream or downstream relative to the immunostimulatory domain. The term "upstream" is used to refer to positions on the 5' side of the immunostimulatory dinucleotide or dinucleotide analog (Y-Z).
 10 The term "downstream" is used to refer to positions on the 3' side of Y-Z.

For example, an immunostimulatory oligonucleotide analog could have the structure

5'-U9-U8-U7-U6-U5-U4-U3-U2-U1-X1-X2-Y-Z-X3-X4-N-N-N-3'

wherein U9-U1 represents an upstream potentiation domain, wherein each U independently represents the same or a different nucleoside immunostimulatory moiety, N represents any nucleoside and X1-X4, Y and Z are as before.
 15

Alternatively, an immunostimulatory oligonucleotide analog could have the structure

5'-N-N-X1-X2-Y-Z-X3-X4-D1-D2-D3-D4-D5-D6-D7-D8-3'

wherein D1-D8 represents a downstream potentiation domain, wherein each D independently represents the same or a different nucleoside or immunostimulatory moiety, and all other symbols are as described above.
 20

In these configurations, an immunostimulatory moiety at U6 would be six positions upstream from the immunostimulatory dinucleotide or dinucleotide analog and an immunostimulatory moiety at D4 would be four positions downstream from the immunostimulatory dinucleotide or dinucleotide analog. The term "position" is used rather than "nucleoside", because any of the U or D positions can represent an immunostimulatory moiety which may or may not be a
 25

nucleoside or nucleoside analog. Of course, oligonucleotide analogs can be constructed having both upstream and downstream potentiation domains.

Table 2 shows representative positions and structures of immunostimulatory moieties within an immunostimulatory oligonucleotide having an upstream potentiation domain. See Figure 7 for definitions of Spacer 9 and Spacer 18 as referred to in Tables 2 and 3.

Table 2

Position	TYPICAL IMMUNOSTIMULATORY MOIETY
X2	2-aminobutyl-1,3-propanediol linker
X1	C3-linker, 2-aminobutyl-1,3-propanediol linker, β -L-deoxynucleoside
U1	1',2'-dideoxyribose, C3-linker, 2'-O-methyl-ribonucleoside
U2	1',2'-dideoxyribose, C3-linker, Spacer 18, 3'-deoxynucleoside, nucleoside methylphosphonate, β -L-deoxynucleoside, 2'-O-propargyl-ribonucleoside
U3	1',2'-dideoxyribose, C3-linker, Spacer 9, Spacer 18, nucleoside methylphosphonate, 2'-5' linkage
U2 + U3	1',2'-dideoxyribose, C3-linker, , β -L-deoxynucleoside
U3 + U4	nucleoside methylphosphonate, 2'-O-methoxyethyl-ribonucleoside
U5 + U6	1',2'-dideoxyribose, C3-linker
X1 + U3	1',2'-dideoxyribose

Table 3 shows representative positions and structures of immunostimulatory moieties within an immunostimulatory oligonucleotide having a downstream potentiation domain.

Table 3

Position	TYPICAL IMMUNOSTIMULATORY MOIETY
X3	nucleoside methylphosphonate
X4	nucleoside methylphosphonate, 2'-O-methyl-ribonucleoside
D1	1',2'-dideoxyribose, nucleoside methylphosphonate
D2	1',2'-dideoxyribose, C3-linker, Spacer 9, Spacer 18, 2-aminobutyl-1,3-propanediol -linker, nucleoside methylphosphonate, β -L-deoxynucleoside
D3	3'-deoxynucleoside, 2'-O-propargyl-ribonucleoside, 2'-5'-linkage
D2 + D3	1',2'-dideoxyribose, β -L-deoxynucleoside

In another embodiment of the invention, the oligonucleotide according to the invention has one or two accessible 5' ends. The present inventors have discovered that immunostimulatory moieties in the region 5' to the immunostimulatory dinucleotide have a greater impact on immunostimulatory activity than do similar substitutions in the region 3' to the immunostimulatory dinucleotide. This observation suggests that the 5'-flanking region of CpG-PS-oligos plays an important role in immunostimulatory activity. Moreover, the inventors have discovered that compounds having two oligonucleotide units attached by way of a 3'-5' or 3'-3' linkage have greater immunostimulatory activity than do compounds in which the two oligonucleotide units are attached by way of a 5'-5' linkage. In some preferred embodiments, therefore, the immunostimulatory oligonucleotide according to the invention comprises a 3'-3' linkage. In some such embodiments, the oligonucleotides have one or two accessible 5' ends.

In a second aspect, the invention provides methods for modulating the immunostimulatory effect of an immunostimulatory oligonucleotide. In some embodiments, the method comprises introducing into the immunostimulatory domain a dinucleotide analog that includes a non-naturally occurring pyrimidine base, as described above for the first aspect of the invention. In some embodiments, the method comprises introducing into the immunostimulatory

domain and/or potentiation domain an immunostimulatory moiety at a specified position, as described above. In some embodiments, the method comprises introducing into the oligonucleotide a 3'-3' linkage.

For purposes of the invention, "introducing an immunostimulatory moiety" at a specified position simply means synthesizing an oligonucleotide that has an immunostimulatory moiety at the specified position. For example, "introducing an immunostimulatory moiety into position U6" simply means synthesizing an oligonucleotide that has an immunostimulatory moiety at such a position, with reference to, *e.g.*, the following structure:

5'-U9-U8-U7-U6-U5-U4-U3-U2-U1-X1-X2-Y-Z-X3-X4-D1-D2-D3-3'.

Preferably, the methods according to this aspect of the invention include introducing an immunostimulatory moiety at a position in the immunostimulatory domain or in an upstream or downstream potentiation domain according to the preferred substitution patterns described in Tables 1-3.

The methods according to this aspect of the invention can be conveniently carried out using any of the well-known synthesis techniques by simply using an appropriate immunomodulatory moiety monomer synthon in the synthesis process in an appropriate cycle to obtain the desired position. Preferred monomers include phosphoramidites, phosphotriesters and H-phosphonates. PS-oligos are readily synthesized, *e.g.*, using β -cyanoethylphosphoramidite chemistry on CPG solid support using appropriate phosphoramidites, deprotected as required, purified by C_{18} reverse phase HPLC, dialyzed against distilled water and lyophilized. The purity of each PS-oligo is readily determined by CGE and the molecular weight can be confirmed by MALDI-TOF mass spectral analysis.

In a third aspect, the invention provides methods for generating an immune response in a patient, such methods comprising administering to the

patient an oligonucleotide analog immunostimulatory compound according to the invention.

In the methods according to this aspect of the invention, preferably, administration of compounds is parenteral, oral, sublingual, transdermal, topical, intranasal, intratracheal, intravaginal, or intrarectal. Administration of the therapeutic compositions can be carried out using known procedures at dosages and for periods of time effective to reduce symptoms or surrogate markers of the disease. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of oligonucleotide from about 0.001 micromolar to about 10 micromolar. For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. Preferably, a total dosage of oligonucleotide will range from about 0.1 mg oligonucleotide per patient per day to about 40 mg oligonucleotide per kg body weight per day. It may be desirable to administer simultaneously, or sequentially a therapeutically effective amount of one or more of the therapeutic compositions of the invention to an individual as a single treatment episode. In some instances, dosages below the above-defined ranges may still provide efficacy. In a preferred embodiment, after the composition of matter is administered, one or more measurement is taken of biological effects selected from the group consisting of complement activation, mitogenesis and inhibition of thrombin clot formation.

In certain preferred embodiments, compounds according to the invention are administered in combination with antibiotics, antigens, allergens, vaccines, antibodies, cytotoxic agents, antisense oligonucleotides, gene therapy vectors, DNA vaccines and/or adjuvants to enhance the specificity or magnitude of the immune response. Either the compound or the vaccine, or both may optionally be linked to an immunogenic protein, such as keyhole limpet hemocyanin, cholera toxin B subunit, or any other immunogenic carrier protein. Any of a plethora of adjuvants may be used, including, without limitation, Freund's complete adjuvant, monophosphoryl lipid A (MPL), saponins, including QS-21, alum, and combinations thereof. Certain preferred embodiments of the methods according

to the invention induce cytokines by administration of immunostimulatory oligonucleotide compounds. In certain embodiments the immunostimulatory oligonucleotide compounds are conjugated to an antigen, hapten, or vaccine. As discussed above, the present inventors have discovered that an accessible 5' end is
5 important to the activity of certain immunostimulatory oligonucleotide compounds. Accordingly, for optimum immunostimulatory activity, the oligonucleotide preferably is conjugated to an antigen or vaccine by means of the 3'-end of oligonucleotide compound.

For purposes of this aspect "in combination with" means in the course of
10 treating the same disease in the same patient, and includes administering the oligonucleotide and/or the vaccine and/or the adjuvant in any order, including simultaneous administration, as well as temporally spaced order of up to several days apart. Such combination treatment may also include more than a single administration of the oligonucleotide, and/or independently the vaccine, and/or
15 independently the adjuvant. The administration of the oligonucleotide and/or vaccine and/or adjuvant may be by the same or different routes.

The method according to this aspect of the invention is useful for model studies of the immune system, and is further useful for the therapeutic treatment of human or animal disease.

20 In a fourth aspect, the invention provides methods for therapeutically treating a patient having disease caused by a pathogen, such methods comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to the invention. Administration is carried out as described for the third aspect of the invention.

25 In a fifth aspect, the invention provides methods for treating a cancer patient, such methods comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to the invention. Administration is carried out as described for the third aspect of the invention.

In a sixth aspect, the invention provides methods for treating autoimmune disorders, such as autoimmune asthma, such methods comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to the invention. Administration is carried out as described for the third aspect of
5 the invention.

In a seventh aspect, the invention provides methods for treating airway inflammation or allergies, such methods comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to the invention. Administration is carried out as described for the third aspect of the
10 invention.

The following examples are intended to further illustrate certain preferred embodiments of the invention, and are not intended to limit the scope of the invention.

EXAMPLES

Example 1: Synthesis of oligonucleotides containing immunomodulatory moieties

Oligonucleotides were synthesized on a 1 micromolar scale using an automated DNA synthesizer (Expedite 8909, PerSeptive Biosystems, Foster City, CA). Standard deoxynucleoside phosphoramidites are obtained from PerSeptive Biosystems. 1',2'-dideoxyribose phosphoramidite, propyl-1-phosphoramidite, 2'-deoxy-5-nitroindole-ribofuranosyl phosphoramidite, 2'-deoxy-uridine phosphoramidite, 2'-deoxy-P phosphoramidite, 2'-deoxy-2-aminopurine phosphoramidite, 2'-deoxy-nebularine phosphoramidite, 2'-deoxy-7-deazaguanosine phosphoramidite, 2'-deoxy-4-thiouridine phosphoramidite, 2'-deoxy-isoguanosine phosphoramidite, 2'-deoxy-5-methylisocytosine phosphoramidite, 2'-deoxy-4-thiothymidine phosphoramidite, 2'-deoxy-K-phosphoramidite, 2'-deoxy-2-aminoadenosine phosphoramidite, 2'-deoxy-N4-ethyl-cytosine phosphoramidite, 2'-deoxy-6-thioguanosine phosphoramidite, 2'-deoxy-7-deaza-xanthosine phosphoramidite, 2'-deoxy-8-bromoguanosine phosphoramidite, 2'-deoxy-8-oxoguanosine phosphoramidite, 2'-deoxy-5-hydroxycytosine phosphoramidite, arabino-cytosine phosphoramidite and 2'-deoxy-5-propynecytosine phosphoramidite were obtained from Glen Research (Sterling, VA). 2'-Deoxy-inosine phosphoramidite were obtained from ChemGenes (Ashland, MA).

Normal coupling cycles or a coupling cycle recommended by the phosphoramidite manufacturer were used for all phosphoramidites. Beaucage reagent was used as an oxidant to obtain phosphorothioate modification. After synthesis, oligonucleotides were deprotected by incubating CPG-bound oligonucleotide with concentrated ammonium hydroxide solution for 1.5-2 hours at room temperature and then incubating the ammonium hydroxide supernatant for 12 hours at 55 degrees C or as recommended by phosphoramidite manufacturer. The ammonium hydroxide solution was evaporated to dryness in

a speed-vac and 5'-DMTr-oligonucleotides were purified by HPLC on a C18 reverse-phase matrix using a solvent system of 0.1 M ammonium acetate and 1:5 ratio 0.1 M ammonium acetate in acetonitrile. Then the oligonucleotides were treated with 80% acetic acid to remove the DMTr group, converted to sodium form and desalted by dialysis against double distilled water. Oligonucleotides were filtered through 0.4 μ filters, lyophilized and redissolved in double distilled water. Characterization was achieved by denaturing PAGE and MALDI-TOF mass spectrometry.

Example 2: Synthesis of CpG-PS-oligos containing cytosine analogs

Following the procedures outlined in Example 1, the following oligonucleotides were synthesized:

Oligo #	Sequence (5'→ 3') and Modification ^a
1	d(CTATCTGACGTTCTCTGT)
2	d(CTATCTGAC C *GTTCTCTGT)
3	d(CTATCTGACC C *TTCTCTGT)
4	d(CTATCTGAC C *GTTCTCTGT)
5	d(CTATCTGACC C *TTCTCTGT)

^a CpG-motif is shown in bold. **C*** represents 5-hydroxycytosine (oligos 2 and 3) or N4-ethylcytosine (oligos 4 and 5).

The oligonucleotides were characterized by CGE and MALDI-TOF mass spectrometry (Brucker Proflex III MALDI-TOF mass spectrometer with 337 nm N2 laser). Molecular weights observed and calculated (shown in parentheses) for each oligonucleotide are as follows: Oligo 1, 5704 (5704.8); Oligo 2, 5720 (5720.8); Oligo 3, 5681 (5680.7); Oligo 4, 5733 (5733); Oligo 5, 5694 (5693).

Example 3: Analysis of spleen weights in treated mice

Female BALB/c mice (4-5 weeks, 19-21 g, Charles River, Wilmington, MA) were used in the study. The animals were fed with commercial diet and water *ad lib*. The animals were injected intraperitoneally with 5 or 10 mg/kg dose of immunostimulatory oligonucleotide compound dissolved in sterile PBS. One group of mice received PBS alone to serve as a control (PBS). Four animals were used for each immunostimulatory oligonucleotide compound. Mice were sacrificed 72 h later, spleens were harvested and weighed.

Example 4: Analysis of immunostimulatory oligonucleotide compounds in mouse lymphocyte proliferation assay

Spleens from CD-1, BALB/c, C57BL/6 mouse (4-8 weeks) were used as source of lymphocytes. Single cell suspensions were prepared by gently mincing with the frosted ends of glass slides. Cells were then cultured in RPMI complete medium [RPMI medium supplemented with 10% fetal bovine serum (FBS) (heat-inactivated at 56 °C for 30 min), 50 µM 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine]. The cells were then plated in 96-well dishes at a density of 10⁶ cells/mL in a final volume of 100 µL. Immunostimulatory oligonucleotide compounds or LPS (lipopolysaccharide) were added to the cell culture in 10 µL of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The cells were then set to culture at 37 °C. After 44 h, 1 µCi ³H-uridine (Amersham, Arlington Heights, IL) was added to the culture in 20 µL of RPMI medium, and the cells were pulse-labeled for another 4 h. The cells were harvested by automatic cell harvester (Skatron, Sterling, VA), and the filters were counted by a scintillation counter. The experiments were performed in triplicate.

Example 5: Lymphocyte proliferatory activity of CpG-PS-oligos containing cytosine analogs

The immunostimulatory activity of CpG-PS-oligos 1-5 (Example 4) was studied using a BALB/c mouse lymphocyte proliferation assay. In brief, mouse spleen cells were cultured and incubated with CpG-PS-oligos at 0.1, 0.3, 1.0 and

3.0 µg/mL concentration for 48 hr and cell proliferation was measured by ³H-uridine incorporation.

Figure 23 shows the dose-dependent cell proliferatory activity of oligos 1-5 in mouse lymphocyte cultures. At a dose of 3.0 µg/mL, oligo 1, with natural
5 cytidine, showed a proliferation index of 29.5 ± 2.1 . Oligo 2, in which the cytosine base of the deoxycytidine of the CpG-motif is replaced with a 5-hydroxycytosine, also showed dose-dependent lymphocyte proliferation. A proliferation index of 23.7 ± 2.9 at 3.0 µg/mL dose was observed for oligo 2. PS-Oligo 4, which contained
10 N4-ethyl-cytosine in place of the cytosine base in the CpG-motif, also showed dose-dependent cell-proliferation activity. The proliferation index of 18.7 ± 1.6 observed for oligo 4 at a dose of 3 µg/mL suggests that the presence of a bulky hydrophobic substitution on the 4-amino group of cytosine in a CpG-motif slightly impedes immunostimulatory activity.

Oligo 3, in which 5-hydroxy-deoxycytidine was placed in the
15 deoxyguanosine position instead of the deoxycytidine position of the CpG-motif, showed a proliferation index that was similar to that observed for media control (Figure 23). Similarly, the control Oligo 5 in which deoxyguanosine in the CpG-motif was substituted with N4-ethyldeoxycytidine, showed cell proliferation similar to that of media control.

20 Other oligos, in which cytosine base in the CpG-motif was replaced with 5-methyl-deoxycytosine (2; see Figure 28), 5-methyl-deoxyisocytosine (3), deoxyuridine (5), or deoxy-P-base (7) showed no or insignificant cell proliferatory activity in the same assay system. These results suggest that (i) cell proliferatory activity is maintained when the cytosine base of the CpG motif is replaced with 5-
25 hydroxycytosine or N4-ethylcytosine (Oligos 2 and 4, respectively), but (ii) substitution of the guanine base with these cytosine analogs results in a loss of cell proliferatory activity.

Example 6: Splenomegaly in mice induced by CpG-PS-oligos containing cytosine analogs

To confirm the *in vitro* effects of CpG-PS-oligos, Oligos 1, 2, and 4 (from Example 4) were injected intraperitoneally (ip) to BALB/c mice at a dose of 10 mg/kg and the change in spleen weight was measured as an indicator of the level of immunostimulatory activity of each PS-oligo. The change in spleen weight as a result of treatment with CpG-PS-oligos is presented in Figure 24. Female BALB/c mice (4-6 weeks, 19-21 gm) were divided in to different groups with four mice in each group. Oligonucleotides were dissolved in sterile PBS and administered intraperitoneally to mice at a dose of 10 mg/kg. After 72 hr, mice were sacrificed and spleens were harvested and weighed. Each circle represents the spleen weight of an individual mouse and the + represents the mean spleen weight for each group.

Oligo 1, which has natural deoxycytidine in the CpG-motif, showed about 45% increase in spleen weight at a dose of 10 mg/kg, compared with the control group of mice that received PBS. Oligo 2, which has a 5-hydroxycytosine in place of the cytosine base in the CpG-motif, showed about 35% increase in spleen weight at the same dose. Oligo 4, which has N4-ethylcytosine in place of the cytosine base in the CpG-motif, showed about 34% increase in spleen weight at the same dose compared to the control group. These data confirm the results observed in lymphocyte proliferation assays for these oligos containing modified cytidine analogs in place of deoxycytidine in the CpG-motif.

Example 7: Structure-activity relationships of C*pG-PS-oligos

The presence of a methyl group at the 5-position of cytosine (5-methyl-deoxycytosine, 2 (Figure 28)) in a CpG-motif completely abolishes CpG related immunostimulatory effects of CpG-PS-oligos. Based on the results observed in *in vitro* and *in vivo* experiments we have constructed structure-activity relationships for the PS-oligos containing cytosine analogs.

The replacement of the cytosine base (1) in the CpG-motif with 5-methyl-isocytosine (3) resulted in complete loss of immunostimulatory activity, as is the

case with 5-methylcytosine (2), which could be as a result of switching the keto and amino groups at the 2 and 4-positions, respectively, and/or placing a hydrophobic methyl group at the 5-position of cytosine.

5 Oligo 2, containing a hydrophilic hydroxy substitution at the 5-position of the cytosine in the CpG-motif, showed immunostimulatory activity similar to that of oligo 1, which contains the natural cytosine base. This observation suggests that bulky hydrophilic groups are better tolerated than are hydrophobic groups at the 5-position of cytosine for immunostimulatory activity of CpG-PS-oligos. Perhaps the binding pocket for the CpG-oligos on receptor is hydrophilic in
10 nature and can not accommodate a hydrophobic group at the 5-position of cytosine.

When the cytosine base in the CpG-motif is replaced with uracil (5 (see Figure 28)), in which keto groups are present at both the 2 and 4-positions, no immunostimulatory activity was observed, suggesting that a hydrogen bond
15 donor amino group at the 4-position of cytosine is critical for immunostimulatory activity. When a large hydrophobic ethyl group is placed on 4-amino group of cytosine in a CpG-motif, reduced lymphocyte proliferation and a slightly reduced increase in spleen weight in mice were observed, suggesting that a bulky ethyl group at this position does not interfere with binding of the CpG-PS-oligo to the
20 receptor factors responsible for immunostimulatory activity. In spite of the ethyl substitution, the 4-amino group of N4-ethylcytosine (6) can participate in hydrogen bond formation with an acceptor. The modified pyrimidine base dP, in which the nitrogen group located at the 4-position involved in ring structure formation with the 5-position, and which does not have a hydrogen bond donor
25 amino group at the 4-position, had no mouse lymphocyte proliferation activity in cultures, suggesting that the 4-amino group of cytosine in a CpG-motif is critical for immunostimulatory activity.

In conclusion, the results presented here show that the functional groups at 2, 3, and 4 positions of the cytosine are important for CpG-related
30 immunostimulatory activity. A hydrophobic substitution at the 5-position of

cytosine completely suppresses immunostimulatory activity of a CpG-oligo, while a hydrophilic group at this position is tolerated well. In addition, the immunostimulatory activity of CpG-PS-oligos containing 5-hydroxycytosine or N4-ethylcytosine in place of cytosine in the CpG-motif can be modulated
5 significantly by incorporating appropriate chemical modifications in the 5'-flanking sequence, suggesting that these cytosine analogs in a CpG-motif are recognized as part of an immunostimulatory motif.

Example 8: Synthesis of end-blocked CpG-PS oligonucleotides

The CpG-PS-oligos shown in Figure 17 were synthesized using an
10 automated synthesizer and phosphoramidite approach. Oligo 1 (16-mer) was synthesized using nucleoside-5'- β -cyanoethylphosphoramidites. Oligo 2, a 32-mer, was synthesized using nucleoside-3'- β -cyanoethylphosphoramidites and controlled pore glass support (CPG-solid support) with a 3'-linked nucleoside in which 16-mer sequence of Oligo 1 was repeated twice; therefore, Oligo 2 had two
15 16-mers (Oligo 1) linked by a normal 3'-5'-linkage. Oligo 3, a 32-mer, was synthesized with two 16-mers (Oligo 1) linked by a 5'-5'-linkage, so Oligo 3 had two 3'-ends and no 5'-end. Synthesis of Oligo 3 was carried out in two steps: the first 16-mer was synthesized using nucleoside-3'- β -cyanoethylphosphoramidites and solid support with a 3'-linked nucleoside, and then synthesis of the second
20 16-mer segment was continued using nucleoside-5'- β -cyanoethylphosphoramidites. Oligo 4, a 32-mer, comprised two 16-mers (Oligo 1) linked by a 3'-3'-linkage, so Oligo 4 had two 5'-ends and no 3'-end. Synthesis of Oligo 4 was carried out in two steps: the first 16-mer was synthesized using nucleoside-5'- β -cyanoethylphosphoramidites and solid support with a 5'-linked
25 nucleoside, and the synthesis of the second 16-mer segment was continued using nucleoside-3'- β -cyanoethylphosphoramidites. Synthesis of Oligos 5-8 was carried out by using the same nucleoside- β -cyanoethylphosphoramidites as for Oligos 1-4, respectively. At the end of the synthesis, Oligos 1-8 were deprotected with concentrated ammonia solution, purified by reversed phase HPLC, detritylated,
30 desalted and dialyzed. The purity of each PS-oligo was checked by CGE and the

molecular weight was confirmed by MALDI-TOF mass spectral analysis (Table 1). The sequence integrity and directionality of 5'-CpG motif in Oligos 1-8 were confirmed by recording melting temperatures (T_m s) of the duplexes with their respective DNA complementary strands (5'-AAGGTCGAGCGTTCTC-3' for Oligos 1-4, and 5'-ATGGCGCACGCTGGGAGA-3' for Oligos 5-8). The T_m s of these duplexes were 53.9 ± 0.9 °C (Oligos 1-4), 61.8 °C (Oligo 5), and 58.8 ± 0.6 °C (Oligos 6-8) (note that Oligo 5 was a 18-mer and Oligos 6-8 were 32-mers but not 36-mers).

Example 9: Mouse spleen lymphocyte proliferatory activity of end-blocked CpG-PS oligonucleotides

Immunostimulatory activity of the end-blocked CpG-PS-oligos of Example 8 was studied initially in a lymphocyte proliferation assay. Typically, mouse (Balb-C) spleen lymphocytes were cultured with CpG-PS-oligos at concentrations of 0.1, 1.0, and 10.0 µg/ml for 48 h and cell proliferation was determined by ^3H -uridine incorporation, as described in Example 3. Results are shown in Figure 17

Oligo 1 induced a dose-dependent effect on cell proliferation; at a concentration of 10 µg/ml (~ 2.0 µM), the proliferation index was 5.0 ± 0.32 . Oligo 2, which consisted of two units of Oligo 1 linked by a 3'-5'-linkage, had a proliferation index of 5.8 ± 0.28 at the same dose (~ 1.0 µM). Oligo 3, which consisted of two units of Oligo 1 linked by a 5'-5'-linkage, had a proliferation index of 2.0 ± 0.26 , reflecting a significantly lower immunostimulatory activity than observed with Oligos 1 and 2. Oligo 4, which consisted of two units of Oligo 1 linked by a 3'-3'-linkage, had a proliferation index of 7.2 ± 0.5 , reflecting a greater immunostimulatory activity than observed with Oligos 1 and 2.

Similar results were obtained with Oligos 5-8. Oligo 5 had a proliferation index of 3.9 ± 0.12 . Oligos 6-8, in which two units of Oligo 5 are linked by a 3'-5'-linkage (Oligo 6), 5'-5'-linkage (Oligo 7), and 3'-3'-linkage (Oligo 8) had proliferation indices of 4.9 ± 0.2 , 1.74 ± 0.21 , and 7.7 ± 0.82 , respectively. Comparison of the results obtained with Oligos 6-8 show that Oligos 6 and 8, in which two Oligo 5 sequences were linked by a 3'-5'-linkage or a 3'-3'-linkage had

greater immunostimulatory activity, while Oligo 7, in which two Oligo 5 were linked by a 5'-5'-linkage had significant less immunostimulatory activity, than did Oligo 5.

Based on lymphocyte proliferation results of Oligos 1-8, it is clear that when oligos are linked through their 5'-ends, there is a significant loss of immunostimulatory activity, while if they are linked through their 3'-ends, there is an increase in immunostimulatory activity. It is important to note that 3'-3'-linked oligos have shown substantially greater stability towards degradation by exonucleases than the oligos that contained a free 3'-end, which could also result in increased immunostimulatory activity. The lower immunostimulatory activity of Oligos 3 and 7, in which the 5'-end of oligos is blocked, suggests that accessibility to 5'-end of oligo is essential for immunostimulatory activity of CpG-PS-oligos.

Example 10: Splenomegaly in mice induced by end-blocked CpG-PS oligonucleotides

To confirm the immunostimulatory activity of Oligos 1-8 (Example 8) *in vivo*, a dose of 5 mg/kg of oligonucleotides was injected intraperitoneally to Balb-C mice. The mice were sacrificed 72 hours post-administration, spleens were removed, blotted to dryness, and weighed. Change in spleen weight in treated and untreated mice was used as a parameter for immunostimulatory activity.

Administration 5 mg/kg dose of Oligo 1 caused about 40% increase in spleen weight compared with the control mice that received PBS. Administration of Oligos 2 and 4 also caused about 50% increase in spleen weight. Administration of Oligo 3 caused no difference in spleen weight compared with control mice. These results further support the observation that Oligo 3, in which 5'-end was blocked, had significantly less immunostimulatory activity compared to oligos that had accessible 5'-end. These results were also confirmed with the administration of Oligos 5-8. Administration of Oligos 5, 6, and 8 caused about 40-50% increase in spleen weight, whereas no change in spleen weight was observed following the administration of Oligo 7.

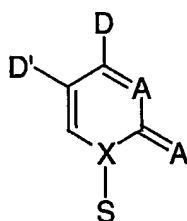
The above results suggest that the immunostimulatory activity of PS-oligos containing a CpG motif is significantly minimized if the 5'-end of the oligo is not accessible. This loss in immunostimulatory activity of Oligos 3 and 7 cannot be explained based on nuclease stability, as both oligos have two 3'-ends and are not
5 more susceptible to 3'-exonuclease degradation than are Oligos 1, 2, 5, and 6, which have one 3'-end. PS-Oligos 4 and 8, which have their 3'-ends blocked and are very stable to degradation by exonucleases, showed similar immunostimulatory activity. Oligos 4 and 8 may show sustained immunostimulatory activity due to their increased *in vivo* stability, which is not
10 evident in the present study as mice were sacrificed at only 72 hours after administration. Studies are in progress in which mice will be sacrificed at times later than 72 hours after administration.

The results described here are intriguing and suggest that the 5'-end of CpG-PS-oligos is critical for immunostimulatory activity. As discussed here, we
15 have shown that substitution of deoxynucleosides in 5'-flanking regions by modified 2'- or 3'-substituted ribonucleosides resulted in increased immunostimulatory activity. In addition, substitution of deoxynucleosides immediately upstream (5'-end) to the CpG motif caused a significant suppression and substitution of deoxynucleosides immediately downstream (3'-end) to the
20 CpG motif had no effect on immunostimulatory activity. Taken together, these results suggest that the enzyme/receptor responsible for the immunestimulation recognizes the CpG motif in oligos from the 5'-end and requires accessibility to the 5'-end.

25 While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

WHAT IS CLAIMED IS:

1. An immunostimulatory oligonucleotide compound, comprising an immunostimulatory dinucleotide of formula 5'-pyrimidine-purine-3', wherein pyrimidine is a non-natural pyrimidine nucleoside and purine is a natural or non-natural purine nucleoside.
2. An immunostimulatory oligonucleotide compound, comprising an immunostimulatory dinucleotide of formula C*pG, wherein C* is a cytidine analog, G is guanosine, 2'-deoxyguanosine, or a guanosine analog, and p is an internucleotide linkage selected from the group consisting of phosphodiester, phosphorothioate, and phosphorodithioate.
3. The immunostimulatory oligonucleotide compound of claim 1, wherein the non-natural pyrimidine nucleoside has the formula (I):



- wherein D is a hydrogen bond donor, D' is selected from the group consisting of hydrogen, hydrogen bond donor, hydrogen bond acceptor, hydrophilic group, hydrophobic group, electron withdrawing group and electron donating group, A is a hydrogen bond acceptor or a hydrophilic group, X is carbon or nitrogen, and S is a pentose or hexose sugar ring, provided that the pyrimidine nucleoside of formula (I) is not cytidine or deoxycytidine.
4. The immunostimulatory oligonucleotide compound of claim 3, wherein the non-natural pyrimidine nucleoside includes a non-naturally occurring pyrimidine base.

5. The immunostimulatory oligonucleotide compound of claim 4, wherein the non-naturally occurring pyrimidine base is selected from the group consisting of 5-hydroxycytosine, 5-hydroxymethylcytosine, N4-alkylcytosine, and 4-thiouracil.

5 6. The immunostimulatory oligonucleotide compound of claim 4, wherein the non-naturally occurring pyrimidine base is selected from the group consisting of 5-hydroxycytosine and N4-ethylcytosine.

7. The immunostimulatory oligonucleotide compound of claim 4, wherein the non-natural pyrimidine nucleoside of formula (I) comprises a non-
10 naturally occurring sugar moiety.

8. The immunostimulatory oligonucleotide compound of claim 7, wherein the non-naturally occurring sugar moiety is arabinose.

9. An immunostimulatory oligonucleotide compound comprising an immunostimulatory domain of formula (II):

15 5'-----X1-X2-Y-Z-X3-X4-----3' (II)

wherein

Y is cytidine, 2'-deoxycytidine, or a non-natural pyrimidine nucleoside;

Z is guanosine, 2'-deoxyguanosine, or a non-natural purine nucleoside;

20 X1 is a naturally occurring nucleoside or an immunostimulatory moiety selected from the group consisting of C3-alkyl linker, 2-aminobutyl-1,3-propanediol linker, and β -L-deoxynucleoside;

X2 is a naturally occurring nucleoside or an immunostimulatory moiety that is an amino linker;

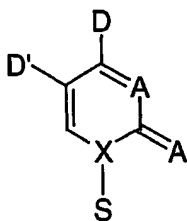
25 X3 is a naturally occurring nucleoside an immunostimulatory moiety that is a nucleoside methylphosphonate;

X4 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of nucleoside methylphosphonate and 2'-O-methylribonucleoside;

provided that at least one of X1, X2, X3, and X4 is an immunostimulatory moiety.

10. The immunostimulatory oligonucleotide compound of claim 9, wherein Y is a non-natural pyrimidine nucleoside.

11. The immunostimulatory oligonucleotide compound of claim 10, wherein Y has the formula (I):



(I)

wherein D is a hydrogen bond donor, D' is selected from the group consisting of hydrogen, hydrogen bond donor, hydrogen bond acceptor, hydrophilic group, hydrophobic group, electron withdrawing group and electron donating group, A is a hydrogen bond acceptor or a hydrophilic group, X is carbon or nitrogen, and S is a pentose or hexose sugar ring, provided that Y is not cytidine or deoxycytidine.

12. An immunostimulatory oligonucleotide compound comprising a sequence of formula (III):

20 5'-Um.....U1-X1-X2-Y-Z-X3-X4-D1.....Dm-3' (III)

wherein:

Y is a non-natural pyrimidine nucleoside;

Z is guanosine, 2'-deoxy-guanosine or a non-natural purine nucleoside;

each X independently is a naturally occurring nucleoside or an immunostimulatory moiety;

wherein U_m -U1 represents an upstream potentiation domain, where each U independently is a naturally occurring nucleoside or an immunostimulatory moiety;

wherein D1- D_m represents a downstream potentiation domain, where each D independently is a naturally occurring nucleoside or an immunostimulatory moiety; and

m , at each occurrence, represents a number from 0 to 30.

10 13. The immunostimulatory oligonucleotide compound of claim 12, wherein at least one X, U, or D is an immunostimulatory moiety.

14. The immunostimulatory oligonucleotide compound of claim 13, wherein:

15 X1 is a naturally occurring nucleoside or an immunostimulatory moiety selected from the group consisting of C3-alkyl linker, 2-aminobutyl-1,3-propanediol linker, and β -L-deoxynucleoside;

X2 is a naturally occurring nucleoside or an immunostimulatory moiety that is an amino linker;

20 X3 is a naturally occurring nucleoside an immunostimulatory moiety that is a nucleoside methylphosphonate;

X4 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of nucleoside methylphosphonate and 2'-O-methylribonucleoside;

25 U1 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of 1',2'-dideoxyribose, C3-linker, and 2'-O-methylribonucleoside;

U2 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of 1',2'-dideoxyribose; C3-linker, Spacer 18, 3'-deoxynucleoside, nucleoside methylphosphonate, β -L-deoxynucleoside, and 2'-O-propargylribonucleoside;

- 5 U3 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of 1',2'-dideoxyribose, C3-linker, Spacer 9, Spacer 18, nucleoside methylphosphonate, and 2'-5' linkage;

- D1 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of 1',2'-dideoxyribose and nucleoside
10 methylphosphonate;

D2 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of 1',2'-dideoxyribose, C3-linker, Spacer 9, Spacer 18, 2-aminobutyl-1,3-propanediol linker, nucleoside methylphosphonate, and β -L-deoxynucleoside; and

- 15 D3 is a naturally occurring nucleoside an immunostimulatory moiety selected from the group consisting of 3'-deoxynucleoside, 2'-O-propargylribonucleoside; and 2'-5' linkage.

15. The immunostimulatory oligonucleotide compound of claim 13, wherein U2 and U3 are both the same immunostimulatory moiety selected from
20 the group consisting of 1',2'-dideoxyribose, C3-linker, or β -L-deoxynucleoside.

16. The immunostimulatory oligonucleotide compound of claim 13, wherein U3 and U4 are both the same immunostimulatory moiety selected from the group consisting of nucleoside methylphosphonate and 2'-O-methoxyethylribonucleoside.

- 25 17. The immunostimulatory oligonucleotide compound of claim 13, wherein U5 and U6 are both the same immunostimulatory moiety selected from the group consisting of 1',2'-dideoxyribose and C3-linker.

18. The immunostimulatory oligonucleotide compound of claim 13, wherein X1 and U3 are both 1',2'-dideoxyribose.

19. The immunostimulatory oligonucleotide compound of claim 13, wherein D2 and D3 are both the same immunostimulatory moiety selected from
5 the group consisting of 1',2'-dideoxyribose and β -L-deoxynucleoside.

20. An immunostimulatory oligonucleotide compound, comprising:
an immunostimulatory dinucleotide of formula 5'-pyrimidine-purine-3',
wherein pyrimidine is a natural or non-natural pyrimidine nucleoside and purine
is a natural or non-natural purine nucleoside;
10 a 3'-3' linkage; and
one or two accessible 5' ends;
provided that the oligonucleotide is not complementary to the *gag* or *tat*
gene of HIV-1.

21. The immunostimulatory oligonucleotide compound of claim 20,
15 which oligonucleotide comprises two accessible 5' ends.

22. The immunostimulatory oligonucleotide compound of claim 20, wherein the immunostimulatory dinucleotide comprises a non-natural pyrimidine nucleoside.

23. A method for modulating the immunostimulatory effect of an
20 immunostimulatory oligonucleotide compound, comprising introducing into the immunostimulatory domain a dinucleotide analog that includes a non-naturally occurring pyrimidine base.

24. A method for modulating the immunostimulatory effect of an immunostimulatory oligonucleotide compound, comprising introducing into the

immunostimulatory domain and/or potentiation domain an immunostimulatory moiety.

25. A method for modulating the immunostimulatory effect of an immunostimulatory oligonucleotide compound, comprising introducing into the
5 oligonucleotide a 3'-3' linkage.

26. A method for generating an immune response in a patient, such method comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to any one of claims 1-22.

27. The method according to claim 26, wherein the oligonucleotide
10 analog immunostimulatory compound is administered in combination with an antibiotic, antigen, allergen, vaccine, antibody, cytotoxic agent, antisense oligonucleotide, gene therapy vector, DNA vaccine, adjuvant, or combination thereof.

28. The method according to claim 26, wherein the immunostimulatory
15 oligonucleotide compound is conjugated to an antigen or vaccine.

29. The method according to claim 28, wherein such conjugation is to the 3'-end of the oligonucleotide compound.

30. A method for therapeutically treating a patient having disease caused by a pathogen, such method comprising administering to the patient an
20 immunostimulatory oligonucleotide compound according to any of claims 1-22.

31. The method according to claim 30, wherein the pathogen is a virus.

32. The method according to claim 30, wherein the pathogen is a parasite.

33. The method according to claim 30, wherein the pathogen is a bacterium.

34. A method for treating a cancer patient, such method comprising administering to the patient an immunostimulatory oligonucleotide compound
5 according to any of claims 1-22.

35. The method according to claim 34, wherein the immunostimulatory oligonucleotide compound is administered in combination with a chemotherapeutic compound.

36. A method for treating an autoimmune disorder, such method
10 comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to any of claims 1-22.

37. The method according to claim 36, wherein the autoimmune disorder is autoimmune asthma.

38. A method for treating airway inflammation or allergy, such method
15 comprising administering to the patient an oligonucleotide analog immunostimulatory compound according to any of claims 1-22.

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

Oligo No.	Sequence & Modification
8	5'-CCTACTAGCGTTCTCATC-3' (133-1)
9	5'-CCTACTAGCXTTCTCATC-3' (139-2)
10	5'-CCTACTAXCGTTCTCATC-3' (133-2)
11	5'-CCTACTXGCGTTCTCATC-3' (139-3)
12	5'-CCTACXAGCGTTCTCATC-3' (133-3)
13	5'-CCTAXTAGCGTTCTCATC-3' (139-4)
14	5'-CCTXCTAGCGTTCTCATC-3' (133-4)
15	5'-CCTXCTAGCCTTCTCATC-3' (145-10a)
16	5'-CCTACTAGCGXTCTCATC-3' (133-5)
17	5'-CCTACTAGCGTXCTCATC-3' (139-7)
18	5'-CCTACTAGCGTTXTCATC-3' (133-6)
19	5'-CCTACTAGCGTTCXCATC-3' (139-8)
20	5'-CCTXXTAGCGTTCTCATC-3' (133-12)
21	5'-XXTAGCGTTCTCATC-3' (139-6)
22	5'-CCTACTAGCGTTCXXATC-3' (139-9)
23	5'-CCTXCTXGCGTTCTCATC-3' (145-10b)

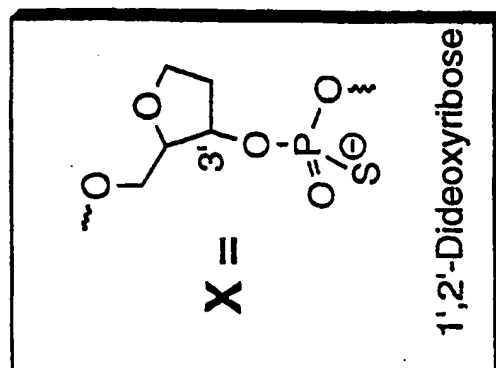


FIG. 1A

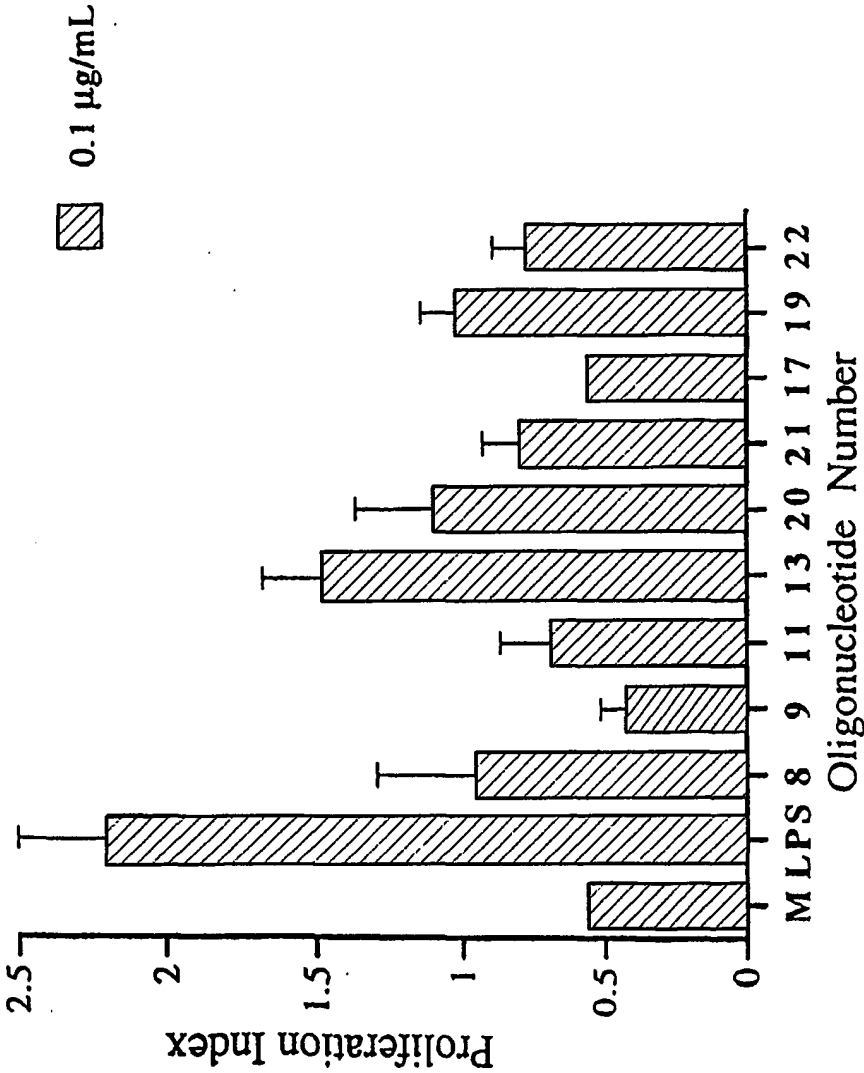


FIG. 1B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

Oligo No.	Sequence & Modification
8	5'-CCTACTAGCGTTCTCATC-3' (133-1)
9	5'-CCTACTAGCXTTCTCATC-3' (139-2)
10	5'-CCTACTAXCGTTCTCATC-3' (133-2)
11	5'-CCTACTXGCGTTCTCATC-3' (139-3)
12	5'-CCTACXAGCGTTCTCATC-3' (133-3)
13	5'-CCTAXTAGCGTTCTCATC-3' (139-4)
14	5'-CCTXCTAGCGTTCTCATC-3' (133-4)
15	5'-CCTXCTAGCCTTCTCATC-3' (145-10a)
16	5'-CCTACTAGCGXTCTCATC-3' (133-5)
17	5'-CCTACTAGCGTXCTCATC-3' (139-7)
18	5'-CCTACTAGCGTTTCTCATC-3' (133-6)
19	5'-CCTACTAGCGTTCXCATC-3' (139-8)
20	5'-CCTXXTAGCGTTCTCATC-3' (133-12)
21	5'-XXTAGCGTTCTCATC-3' (139-6)
22	5'-CCTACTAGCGTTCXXATC-3' (139-9)
23	5'-CCTXCTXGCGTTCTCATC-3' (145-10b)

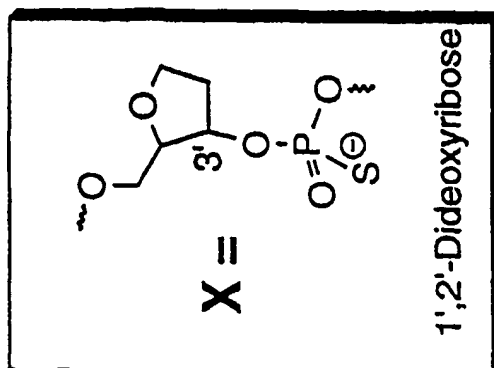


FIG. 2A

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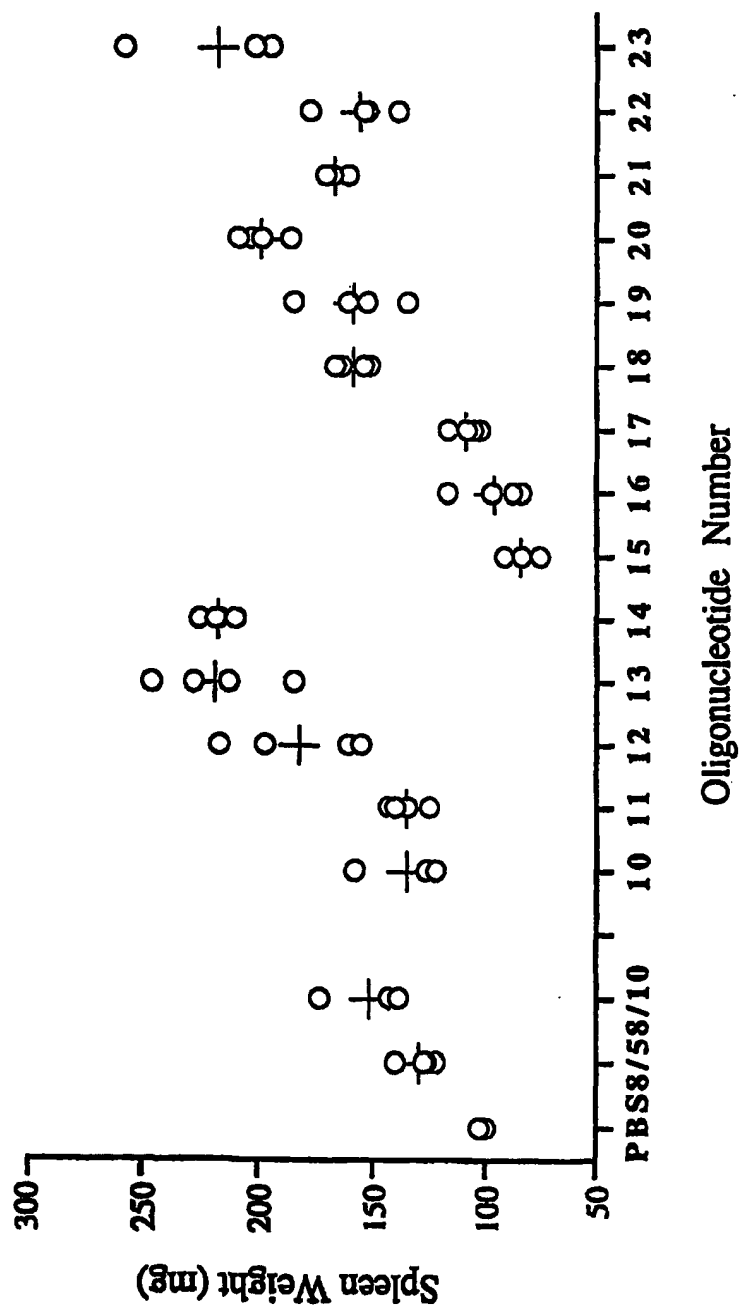


FIG. 2B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTATCTGAXGTTCTCTGT-3' (131-13)
3	5'-CTATCTGXCGTTCTCTGT-3' (131-2)
4	5'-CTATCXGACGTTCTCTGT-3' (131-3)
5	5'-CTAXCTGACGTTCTCTGT-3' (131-4)
6	5'-CTATCTGACGXTCCTCTGT-3' (131-5)
7	5'-CTATCTGACGTTXTCTGT-3' (131-6)

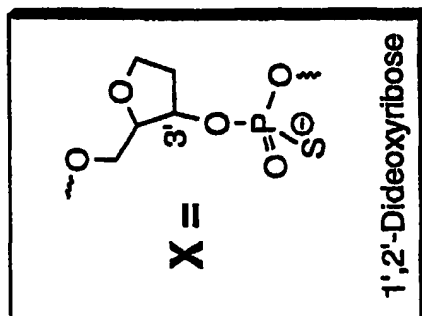


FIG. 3A

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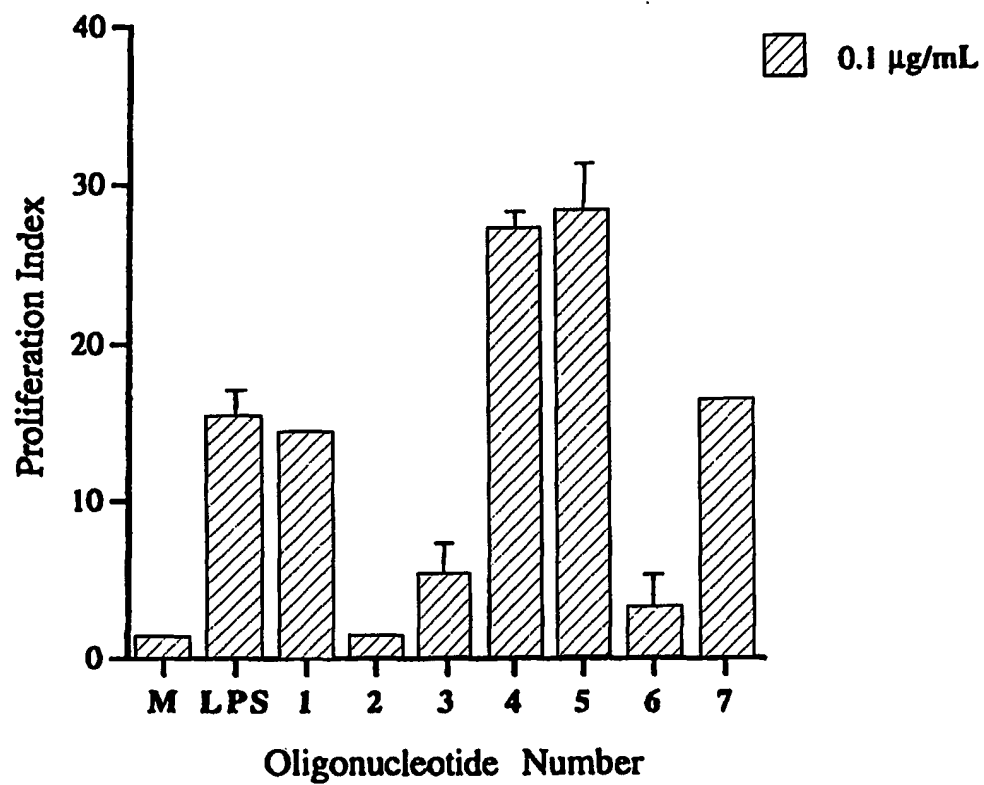


FIG. 3B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

Oligo No.	Sequence & Modification
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTATCTGAXGTTCTCTGT-3' (131-13)
3	5'-CTATCTGXCGTTCTCTGT-3' (131-2)
4	5'-CTATCXGACGTTCTCTGT-3' (131-3)
5	5'-CTAXCTGACGTTCTCTGT-3' (131-4)
6	5'-CTATCTGACGXTCTCTGT-3' (131-5)
7	5'-CTATCTGACGTTXCTGT-3' (131-6)

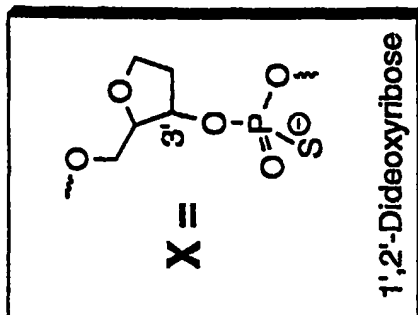


FIG. 4A

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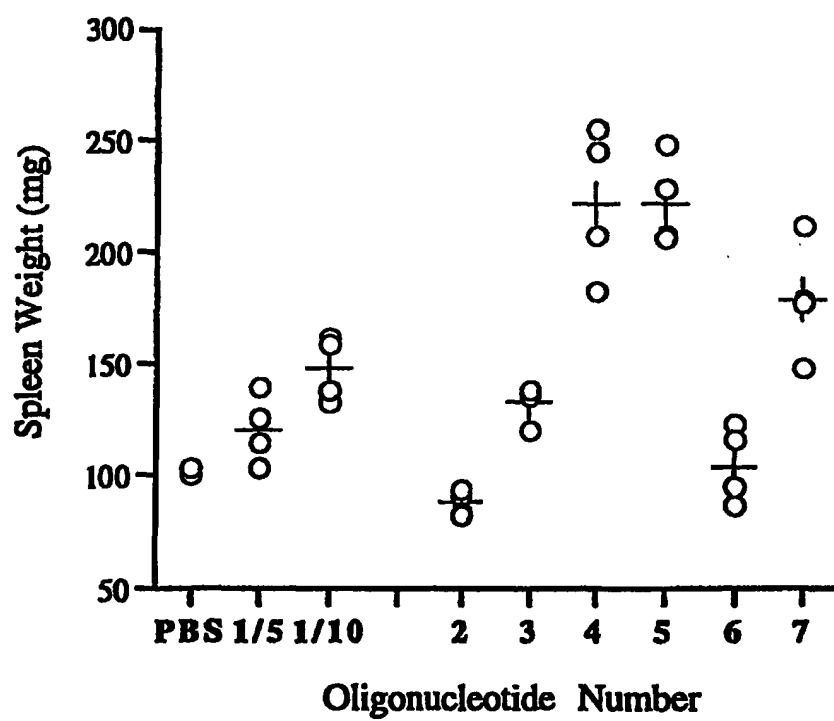


FIG. 4B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
133-1	5'-CCTACTAGCGTTCTCATC-3'
141-2	5'-CCTACTAGCXTTCTCATC-3'
141-3	5'-CCTACTXGCGTTCTCATC-3'
141-4	5'-CCTAXTAGCGTTCTCATC-3'
141-5	5'-CCTXXTAGCGTTCTCATC-3'
141-6	5'-XXTAGCGTTCTCATC-3'
141-7	5'-CCTACTAGCGTXCTCATC-3'
141-8	5'-CCTACTAGCGTTCXCATC-3'
141-9	5'-CCTACTAGCGTTCXXATC-3'
131-1	5'-CTATCTGACGTTCTCTGT-3'
137-2	5'-CTATCTGXCGTTCTCTGT-3'
137-3	5'-CTATCXGACGTTCTCTGT-3'
137-4	5'-CTAXCTGACGTTCTCTGT-3'

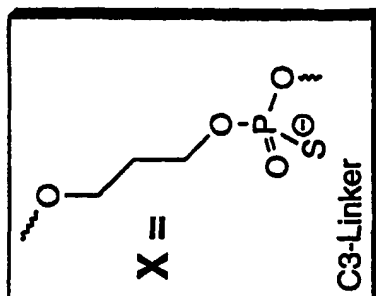


FIG. 5A

10/53

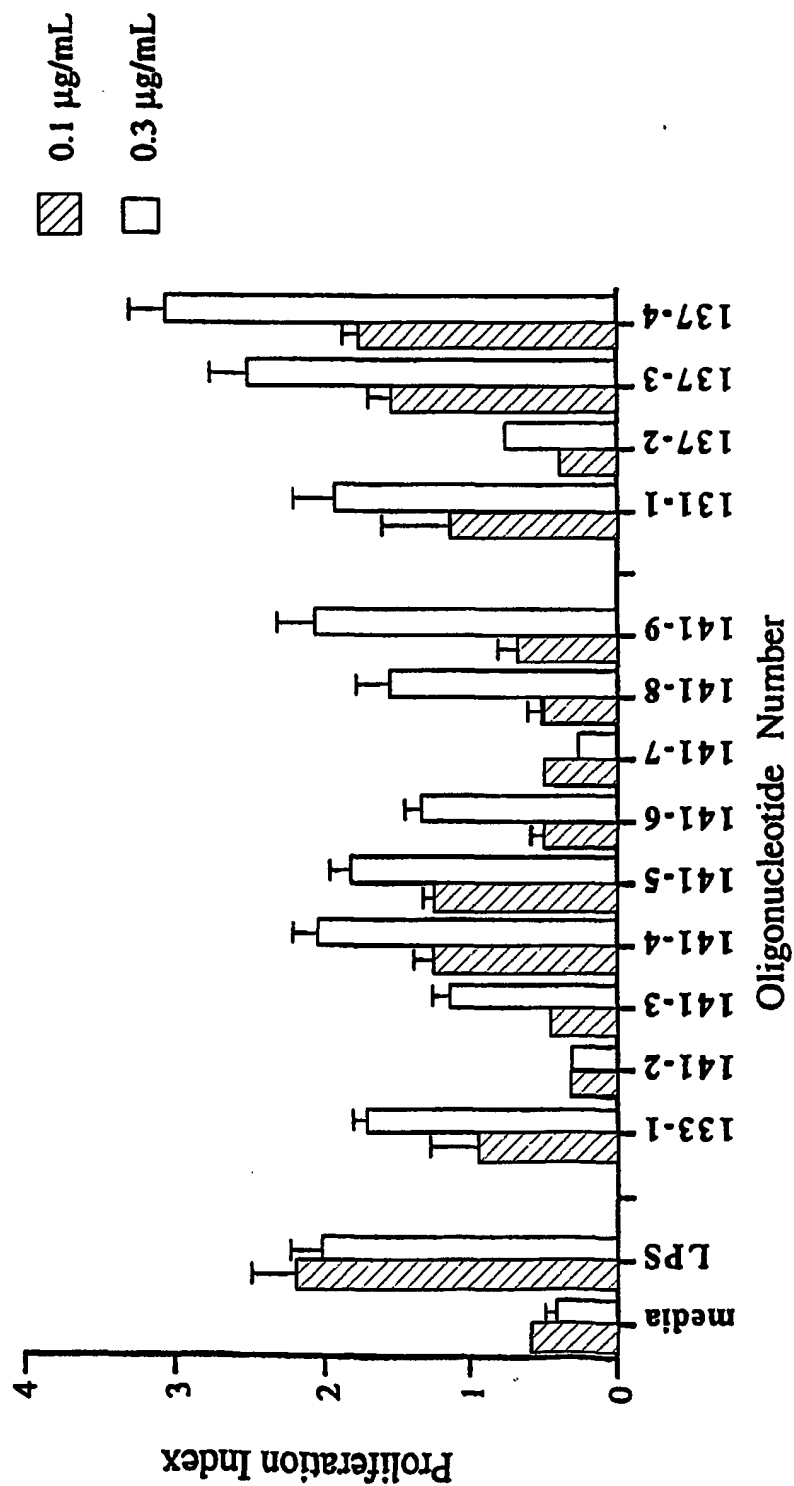


FIG. 5B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
133-1	5'-CCTACTAGCGTTCATC-3'
141-2	5'-CCTACTAGCXTTCTCATC-3'
141-3	5'-CCTACTXGCGTTCATC-3'
141-4	5'-CCTAXTAGCGTTCATC-3'
141-5	5'-CCTXXTAGCGTTCATC-3'
141-6	5'-XXTAGTAGCGTTCATC-3'
141-7	5'-CCTACTAGCGTXCTCATC-3'
141-8	5'-CCTACTAGCGTTCXCATC-3'
141-9	5'-CCTACTAGCGTTCXXATC-3'
131-1	5'-CTATCTGACGTTCTCTGT-3'
137-2	5'-CTATCTGXCGTTCCTCTGT-3'
137-3	5'-CTATCXGACGTTCTCTGT-3'
137-4	5'-CTAXCTGACGTTCTCTGT-3'

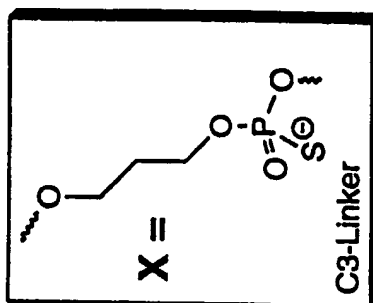


FIG. 6A

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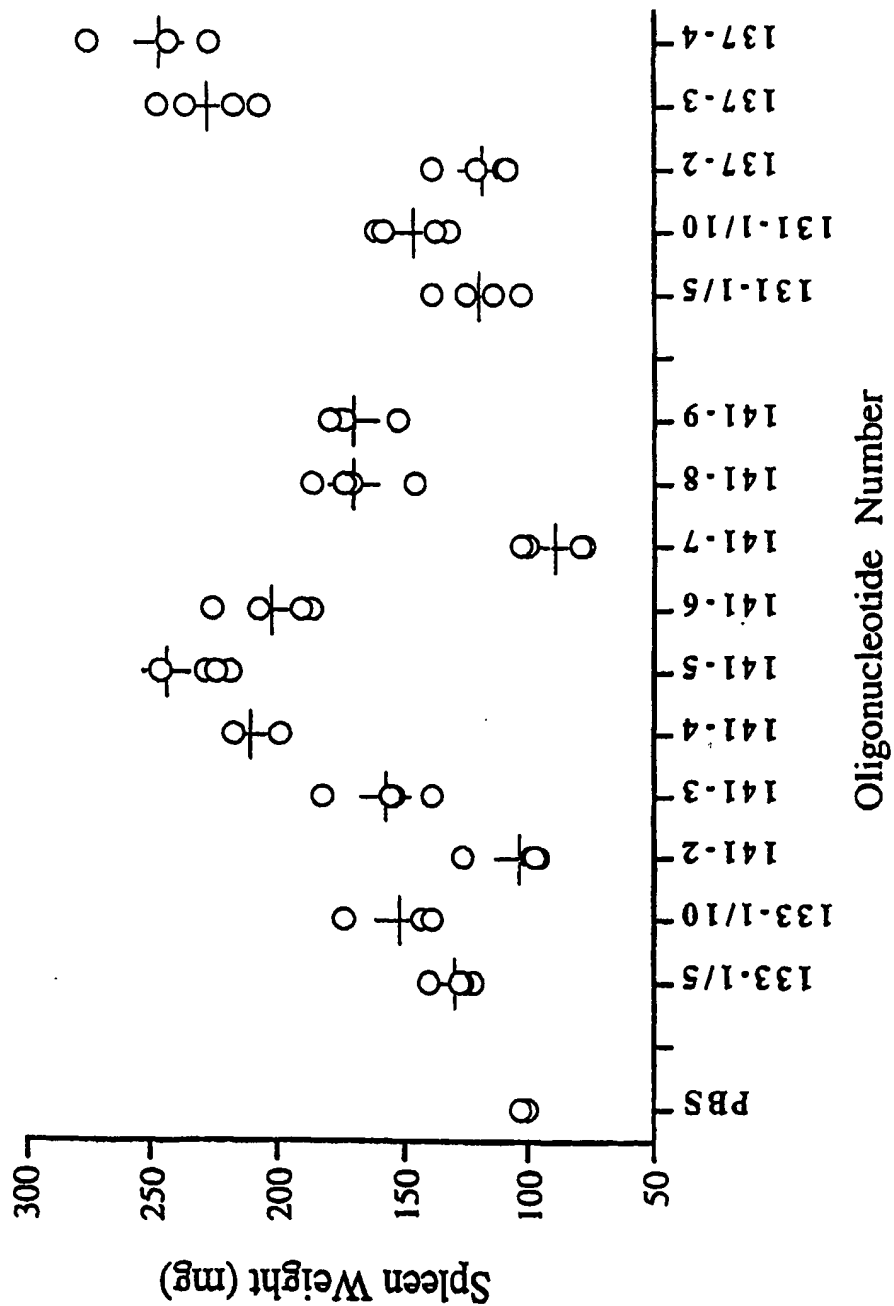


FIG. 6B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTAXCTGACGTTCTCTGT-3' (153-1)
3	5'-CTATCTGACGTTXCXCTGT-3' (153-2)
4	5'-CTAYCTGACGTTCTCTGT-3' (153-3)
5	5'-CTATCTGACGTTCYCTGT-3' (153-4)
6	5'-CCTACTAGCGTTCTCATC-3' (133-1)
7	5'-CCTXCTAGCGTTCTCATC-3' (155-1)
8	5'-CCTACTAGCGTTXCXCATC-3' (155-2)
9	5'-CCTYCTAGCGTTCTCATC-3' (155-3)
10	5'-CCTACTAGCGTTCYCATC-3' (155-4)

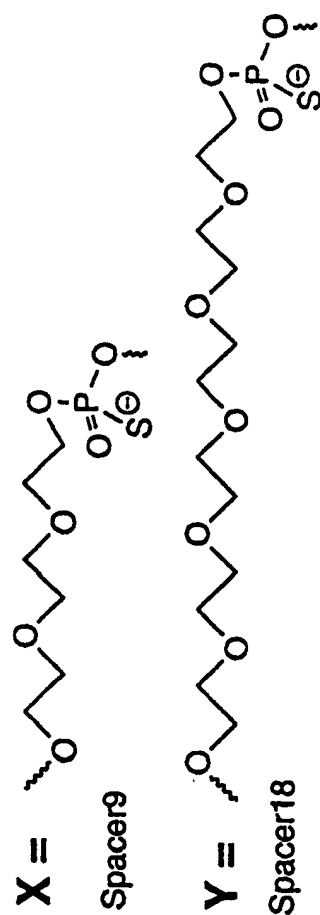


FIG. 7A

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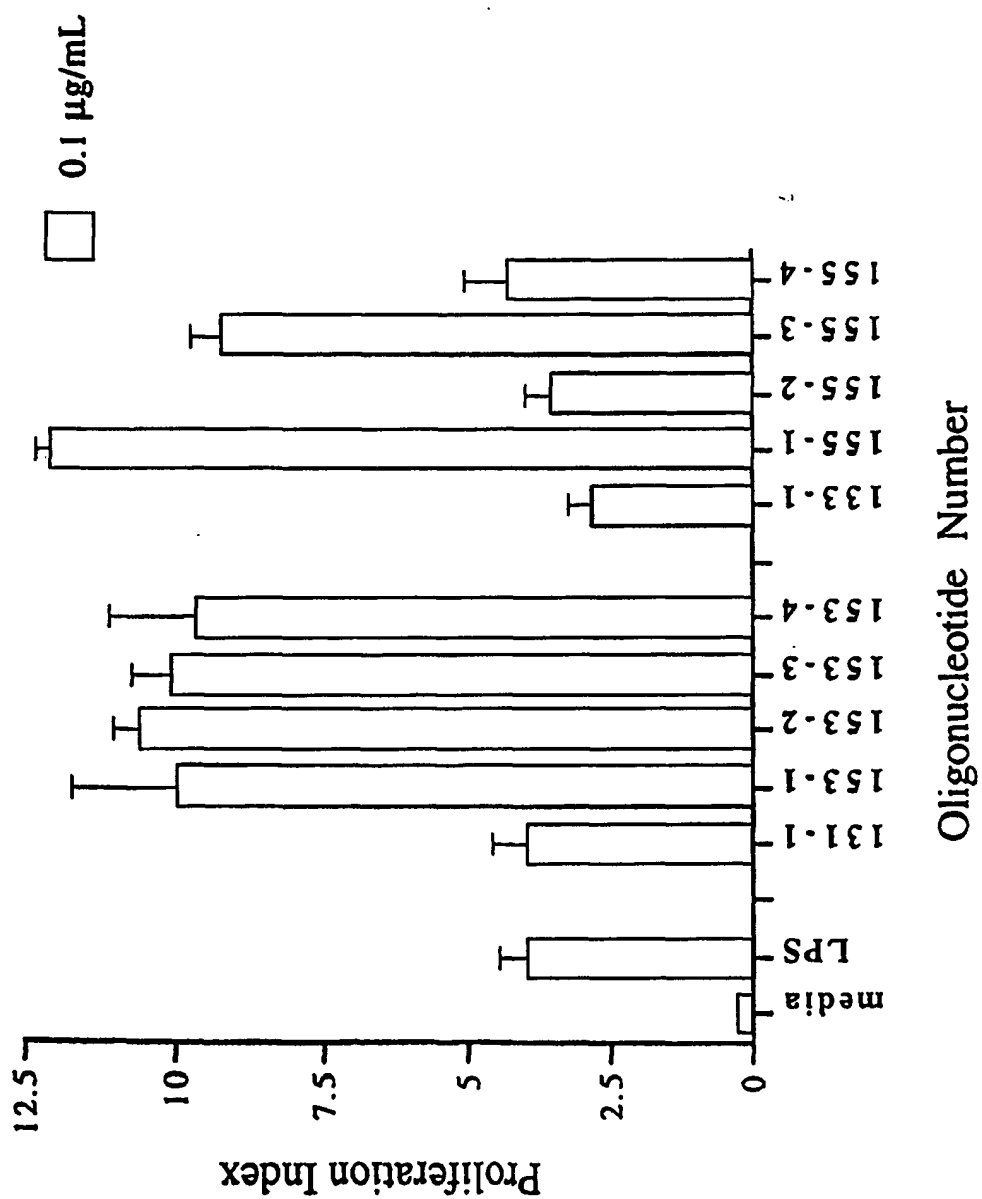


FIG. 7B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTAXCTGACGTTCTCTGT-3' (153-1)
3	5'-CTATCTGACGTTCTCTGT-3' (153-2)
4	5'-CTAYCTGACGTTCTCTGT-3' (153-3)
5	5'-CTATCTGACGTTCTCTGT-3' (153-4)
6	5'-CCTACTAGCGTTCTCATC-3' (133-1)
7	5'-CCTXCTAGCGTTCTCATC-3' (155-1)
8	5'-CCTACTAGCGTTCTCATC-3' (155-2)
9	5'-CCTYCTAGCGTTCTCATC-3' (155-3)
10	5'-CCTACTAGCGTTCTCATC-3' (155-4)

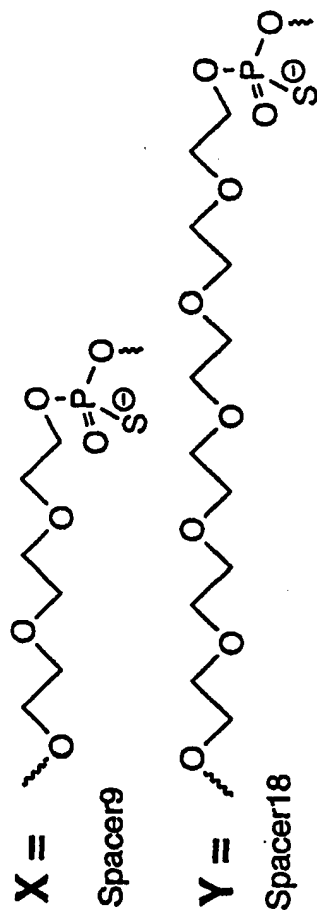


FIG. 8A

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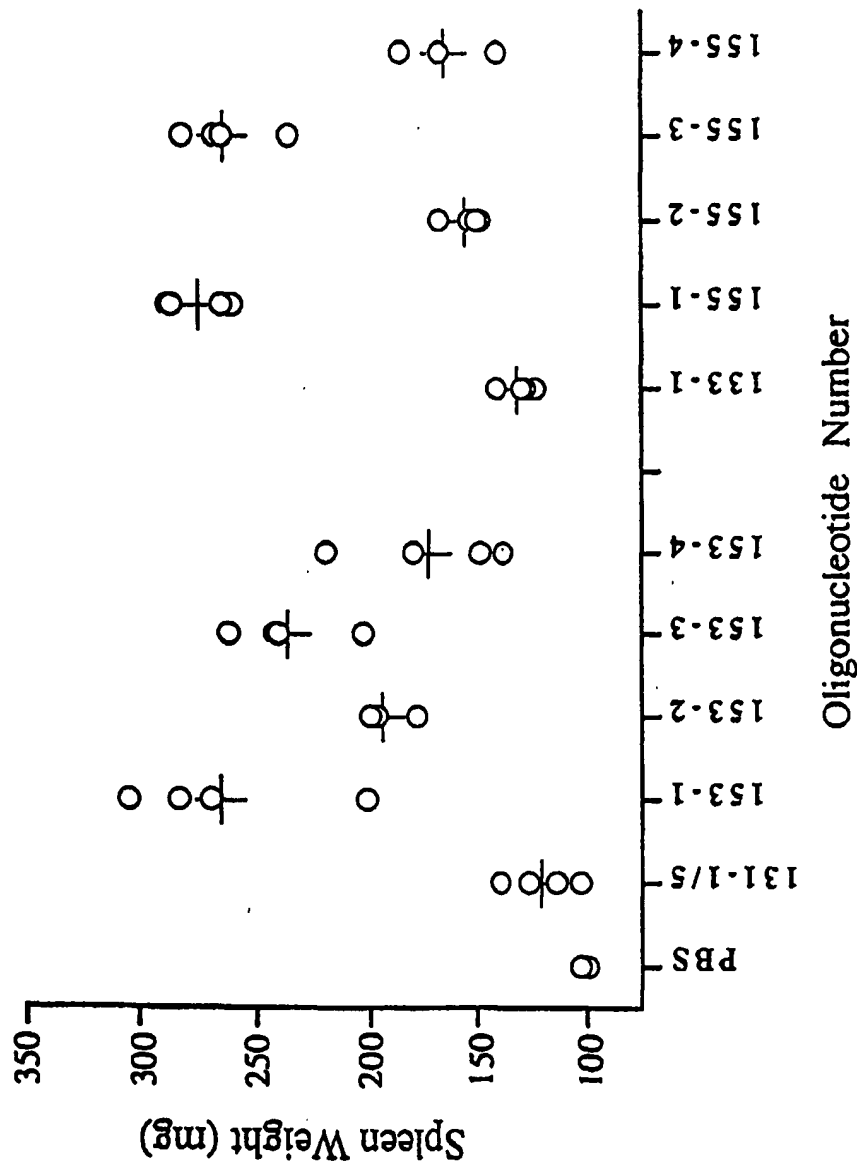


FIG. 8B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTATCTGXC GTTCTCTGT-3' (157-1)
3	5'-CTATCTXACGTTCTCTGT-3' (157-2)
4	5'-CTAXCTGACGTTCTCTGT-3' (157-3)
5	5'-CTATCTGACGTXCTCTGT-3' (157-4)
6	5'-CTATCTGACGTTXCCTGT-3' (157-5)

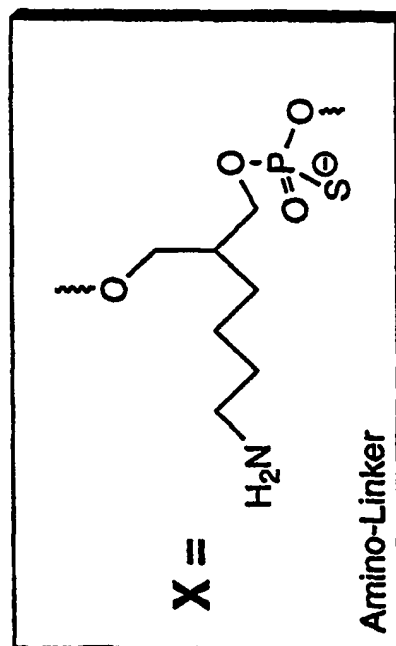


FIG. 9A

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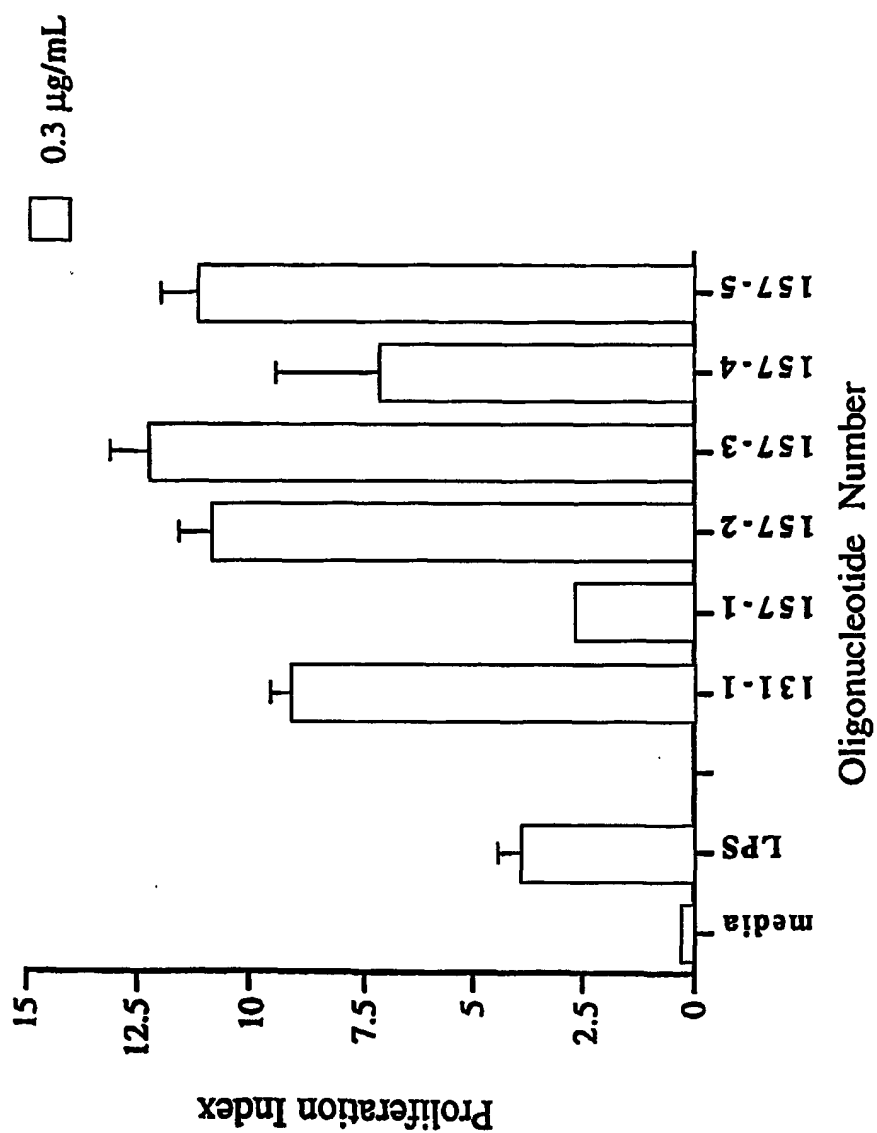


FIG. 9B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTATCTGXCGTTCTCTGT-3' (157-1)
3	5'-CTATCTXACGTTCTCTGT-3' (157-2)
4	5'-CTAXCTGACGTTCTCTGT-3' (157-3)
5	5'-CTATCTGACGTXCTCTGT-3' (157-4)
6	5'-CTATCTGACGTTXCXCTGT-3' (157-5)

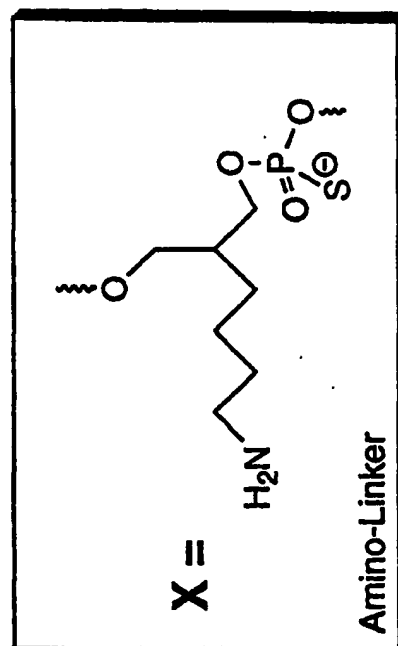


FIG. 10A

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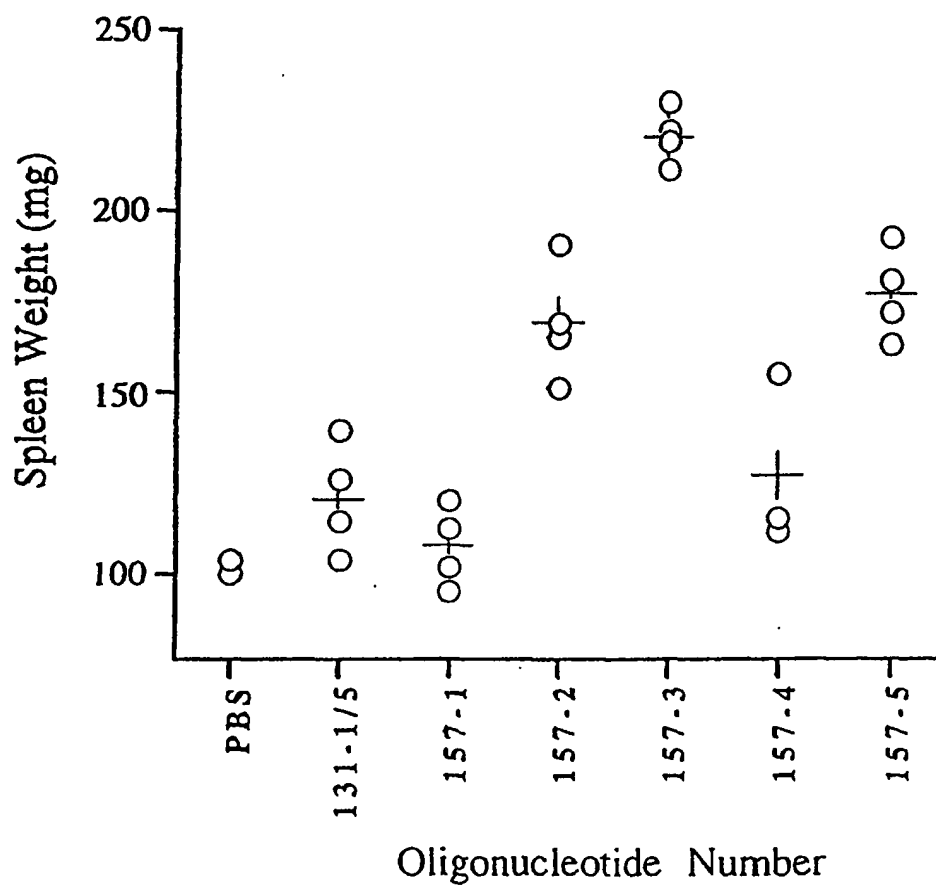


FIG. 10B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

Oligo No.	Sequence & Modification
131-1	5'-CTATCTGACGTTCTCTGT-3'
159-1	5'-CTATCTGACXTTCTCTGT-3'
159-2	5'-CTATCTGAXGTTCTCTGT-3'
159-3	5'-CTATXTGACGTTCTCTGT-3'
159-4	5'-CTATCTGACGTTCTXTGT-3'
133-1	5'-CCTACTAGCGTTCTCATC-3'
161-1	5'-CCTACTAGCXTTCTCATC-3'
161-2	5'-CCTACTAGXGTTCTCATC-3'
161-3	5'-CCTACTAXCGTTCTCATC-3'
161-4	5'-CCTAXTAGCGTTCTCATC-3'
161-5	5'-CCTACTAGCGTTCTXTATC-3'

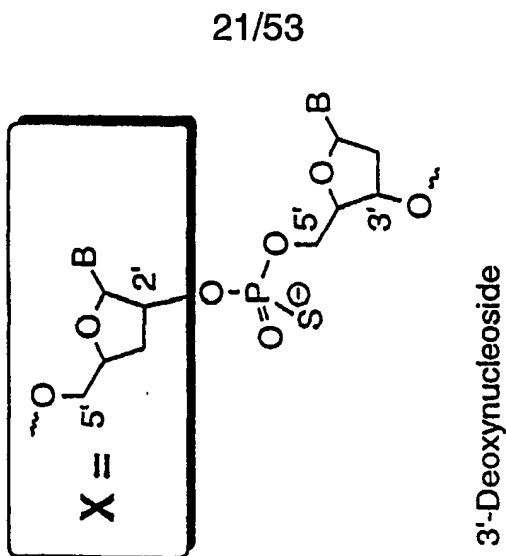


FIG. 11A

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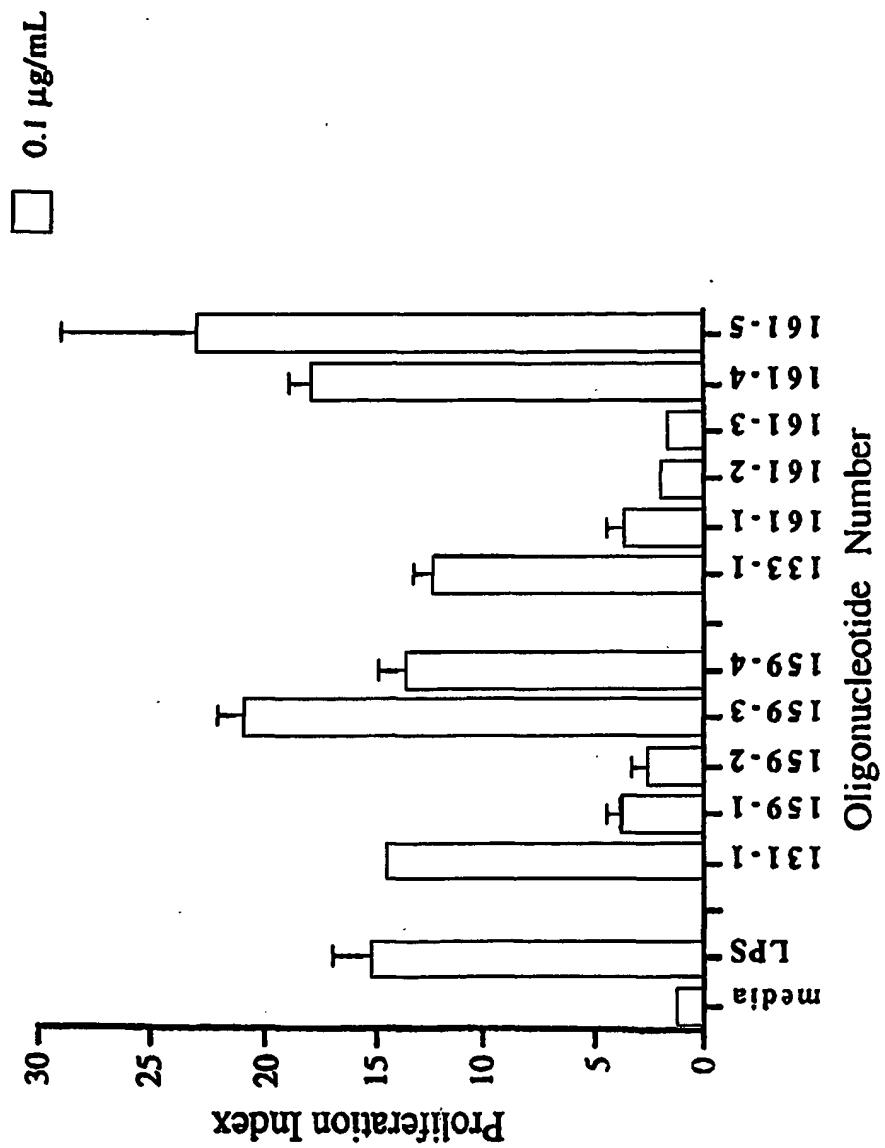


FIG. 11B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

Oligo No.	Sequence & Modification
131-1	5'-CTATCTGACGTTCTCTGT-3'
159-1	5'-CTATCTGACXTTCTCTGT-3'
159-2	5'-CTATCTGAXGTTCTCTGT-3'
159-3	5'-CTATXTGACGTTCTCTGT-3'
159-4	5'-CTATCTGACGTTCTXTGT-3'
133-1	5'-CCTACTAGCGTTCTCATC-3'
161-1	5'-CCTACTAGCXTTCTCATC-3'
161-2	5'-CCTACTAGXGTTCTCATC-3'
161-3	5'-CCTACTAXCGTTCTCATC-3'
161-4	5'-CCTAXTAGCGTTCTCATC-3'
161-5	5'-CCTACTAGCGTTCTXATC-3'

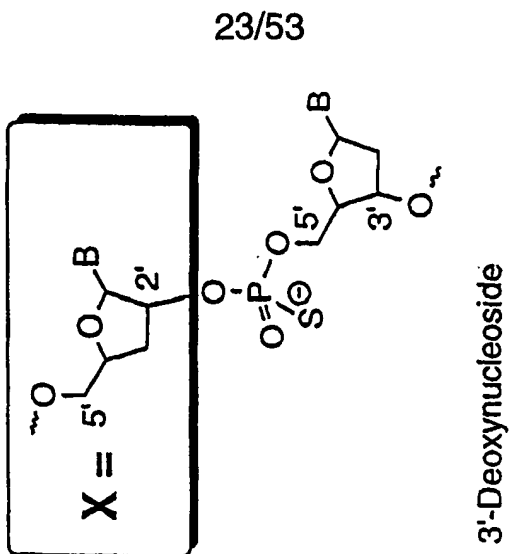


FIG. 12A

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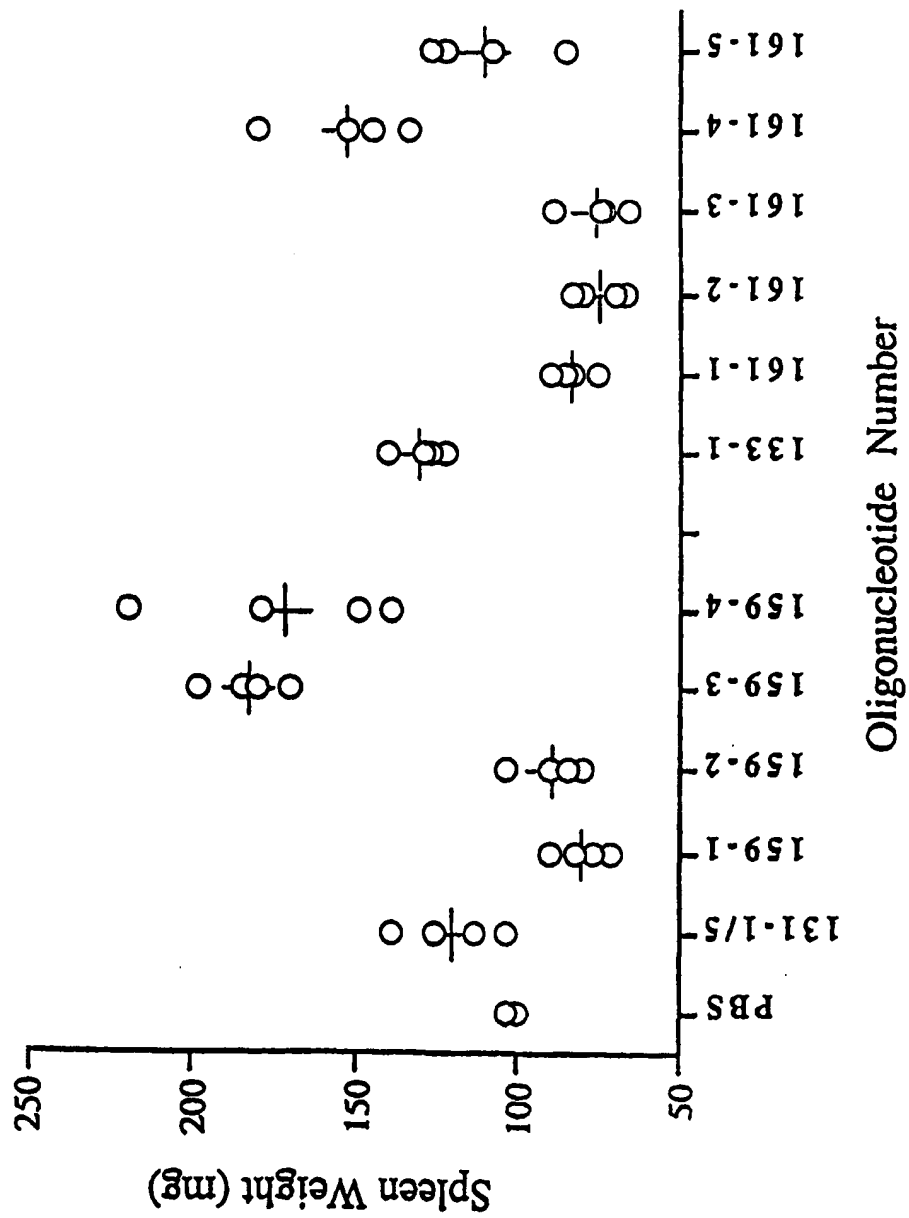


FIG. 12B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

Oligo No.	Sequence & Modification
131-1	5'-CTATCTGACGTTCTCTGT-3'
167-1	5'-CTATCTGXC GTTCTCTGT-3'
167-2	5'-CTATCTXACGTTCTCTGT-3'
167-3	5'-CTATCXGACGTTCTCTGT-3'
167-4	5'-CTATXTGACGTTCTCTGT-3'
167-5	5'-CTAXCTGACGTTCTCTGT-3'
167-6	5'-CTXCTGACGTTCTCTGT-3'
167-7	5'-CTATCTGACGXTCTCTGT-3'
167-8	5'-CTATCTGACGTXCTCTGT-3'
167-9	5'-CTATCTGACGTTXTCTGT-3'
167-10	5'-CTATCTGACGTTXCXCTGT-3'
167-11	5'-CTATCTGACGTTCTXTGT-3'
167-12	5'-CTATCTGACGTTCTXXGT-3'

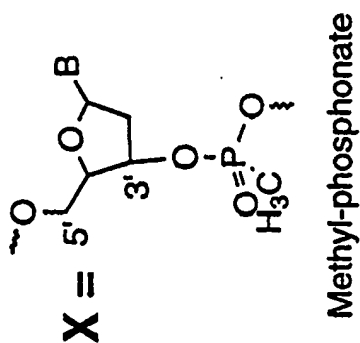


FIG. 13A

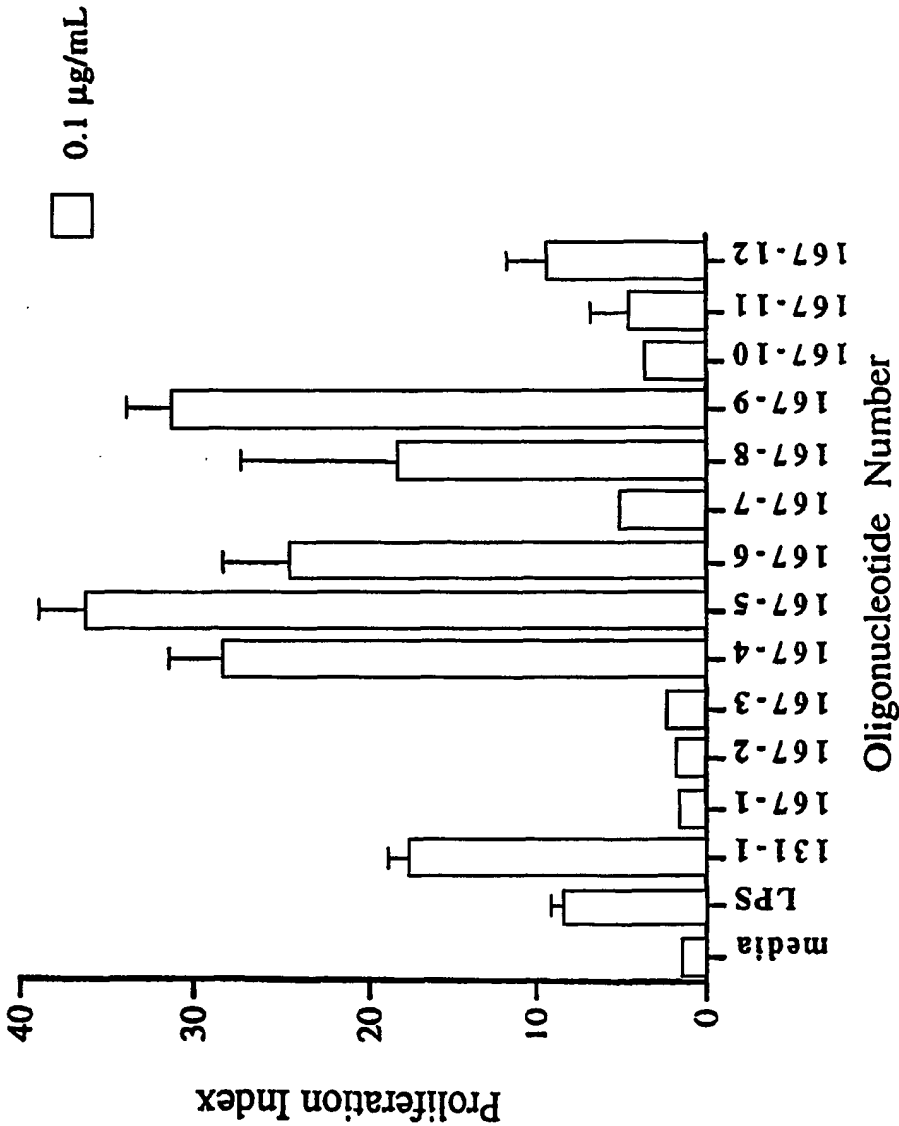


FIG. 13B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
131-1	5'-CTATCTGACGTTCTCTGT-3'
167-1	5'-CTATCTGXCGTTCTCTGT-3'
167-2	5'-CTATCTXACGTTCTCTGT-3'
167-3	5'-CTATCXGACGTTCTCTGT-3'
167-4	5'-CTATXTGACGTTCTCTGT-3'
167-5	5'-CTAXCTGACGTTCTCTGT-3'
167-6	5'-CTXXXCTGACGTTCTCTGT-3'
167-7	5'-CTATCTGACGXTCTCTGT-3'
167-8	5'-CTATCTGACGTXCTCTGT-3'
167-9	5'-CTATCTGACGTTXTCTGT-3'
167-10	5'-CTATCTGACGTTXCCTGT-3'
16711	5'-CTATCTGACGTTCTXTGT-3'
167-12	5'-CTATCTGACGTTCTXXGT-3'

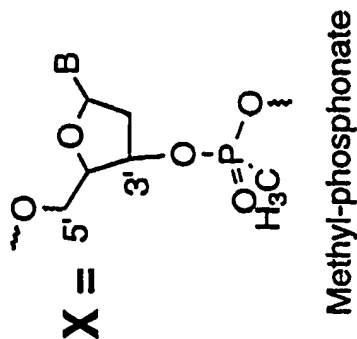


FIG. 14A

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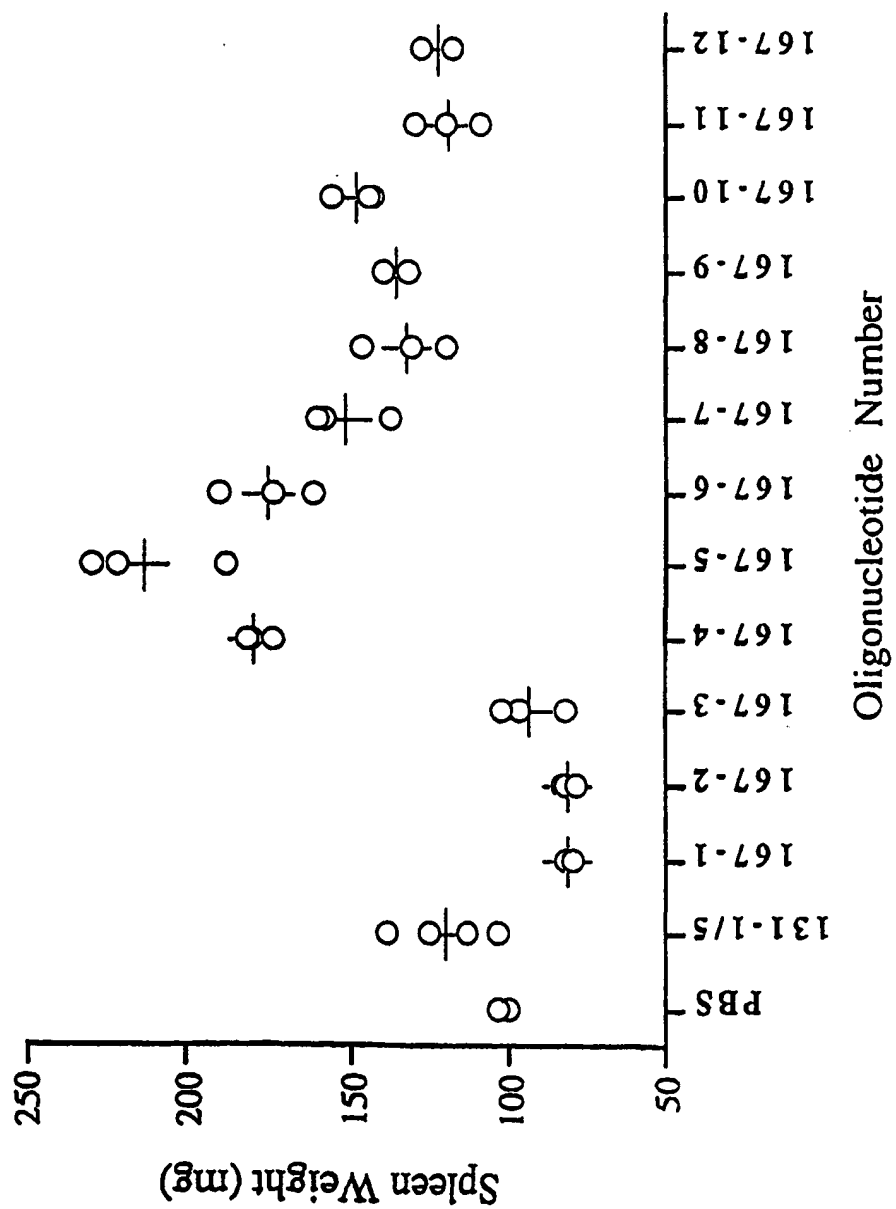


FIG. 14B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
113-1	5'-TCCATGACGTTCCCTGATGC-3'
23-1	5'-TCCATGXCGTTCCCTGATGC-3'
23-3	5'-TCCAXGACGTTCCCTGATGC-3'
29-2	5'-TYATGACGGTCCCTGATGC-3'

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$R_x = 2\text{'-O-Methylribonucleoside}$
 $R_y = 2\text{'-O-Methoxyethoxyribonucleoside}$

FIG. 15A

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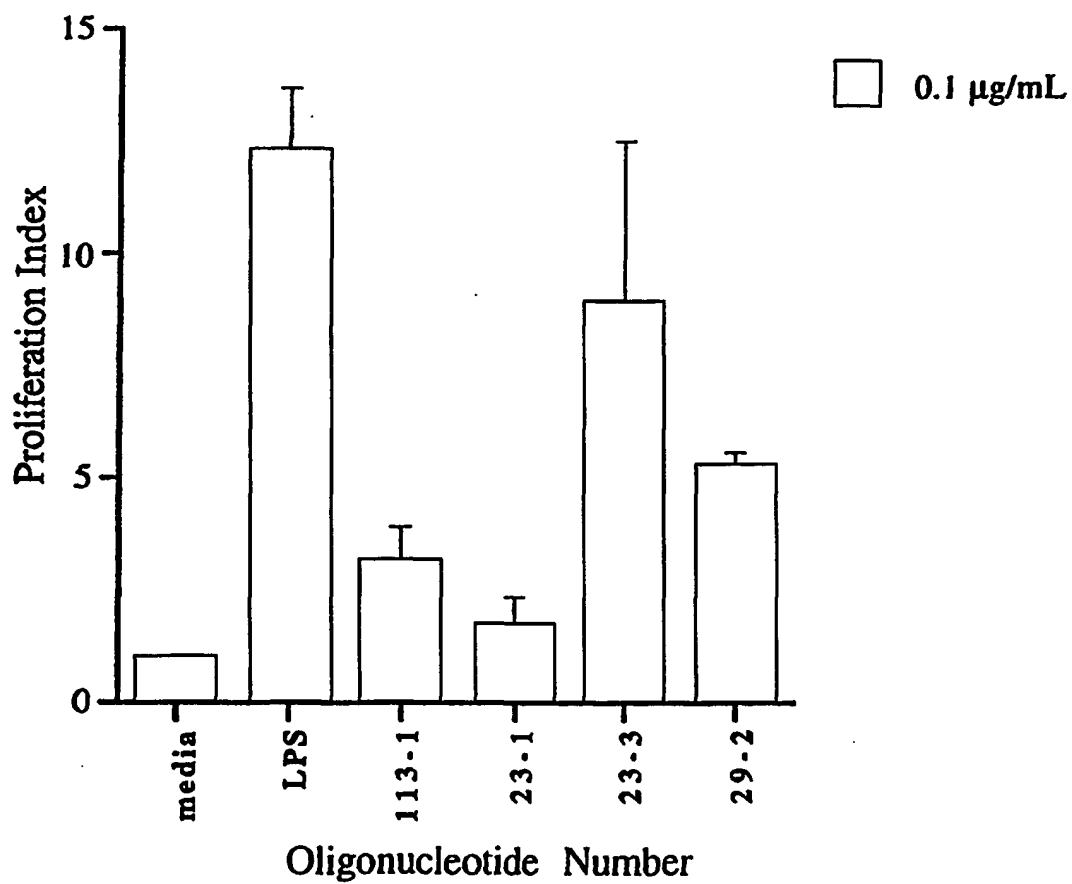
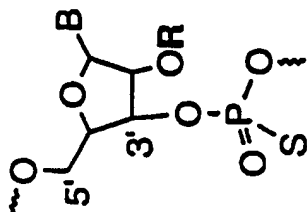


FIG. 15B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
113-1	5'-TCCATGACGTTCCCTGATGC-3'
23-1	5'-TCCATGXCGTTCCCTGATGC-3'
23-3	5'-TCCAXGACGTTCCCTGATGC-3'
29-2	5'-TYVATGACGGTCCTGATGC-3'



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R_x = 2'-O-Methylribonucleoside
 R_y = 2'-O-Methoxyethoxyribonucleoside

FIG. 16A

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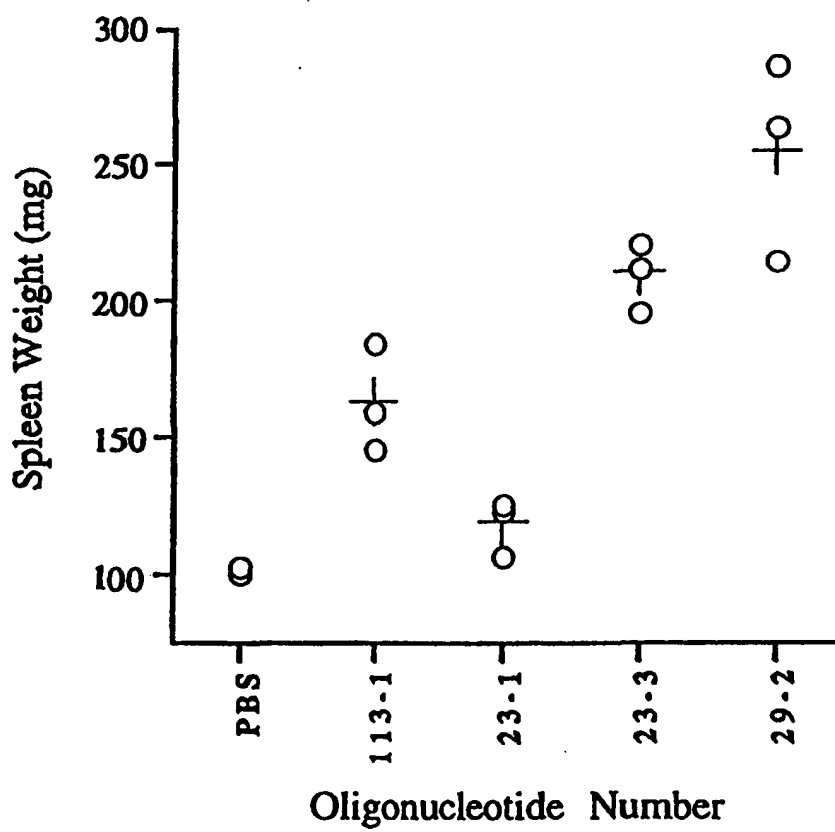


FIG. 16B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-GAGAACGCTCGACCTT-3'
2	5'-GAGAACGCTCGACCTT-3'—5'-GAGAACGCTCGACCTT-3'
3	3'-TTCCAGCTCGCAAGAG-5'—5'-GAGAACGCTCGACCTT-3'
4	5'-GAGAACGCTCGACCTT-3'—3'-TTCCAGCTCGCAAGAG-5'
5	5'-TCTCCAGCGTGCGCCAT-3'
6	5'-TCTCCAGCGTGCGCCAT-3'—5'-TCTCCAGCGTGCGCCAT-3'
7	3'-TACCGCGTGCGACCCCT-5'—5'-TCTCCAGCGTGCGCCAT-3'
8	5'-TCTCCAGCGTGCGCCAT-3'—3'-TACCGCGTGCGACCCCT-5'

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FIG. 17A

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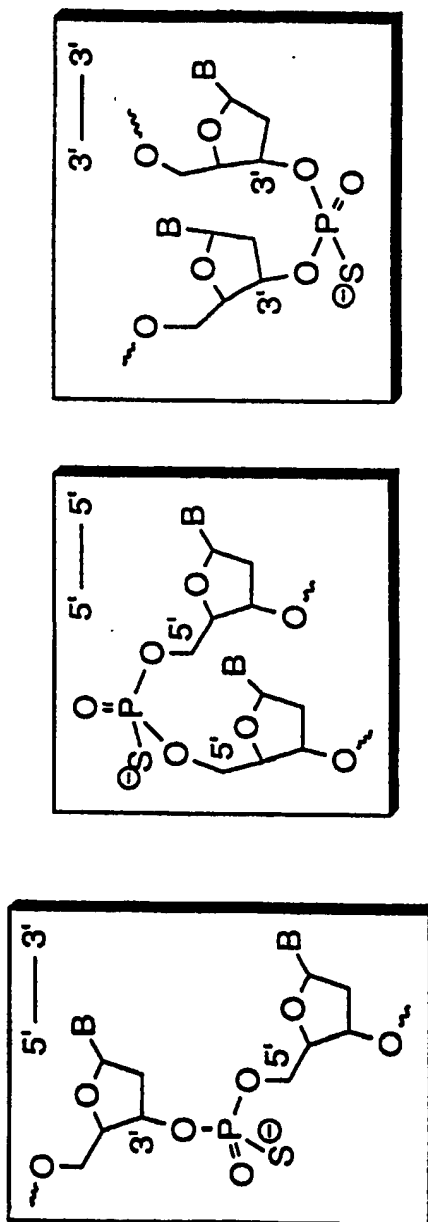


FIG. 17B

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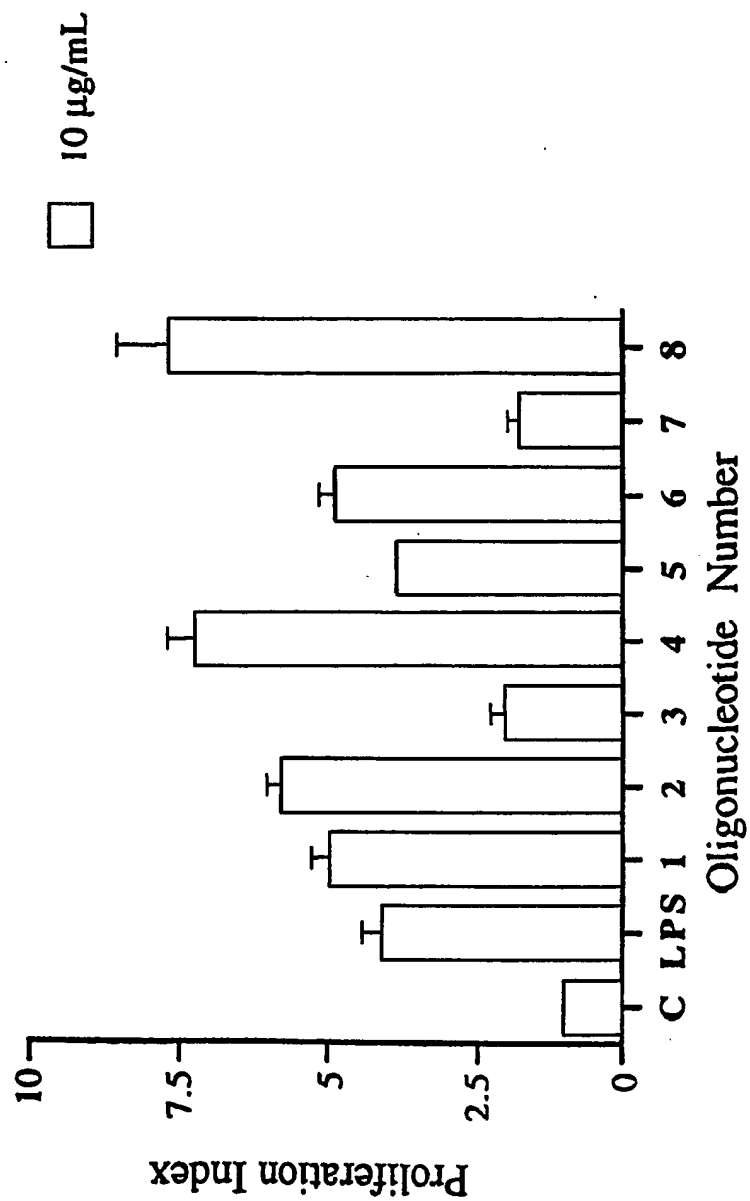


FIG. 17C

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

Oligo No.	Sequence & Modification
131-1	5'-CTATCTGACGTTCTCTGT-3'
175-1	5'-CTATXGACGTTCTCTGT-3'
175-2	5'-CTATCTGACGTTXCXCTGT-3'
175-3	5'-CTAXXGACGTTCTCTGT-3'
175-4	5'-CTATCTGACGTTCCXXGTGT-3'
175-5	5'-CTATCTGAXXTTCTCTGT-3'
175-6	5'-CTATCTXACGTTCTCTGT-3'
175-7	5'-CTATCTGACGTXCCTCTGT-3'
175-9	5'-CTATCTGACGTTCTCTGT-3' (all-β-L-DNA/PS)

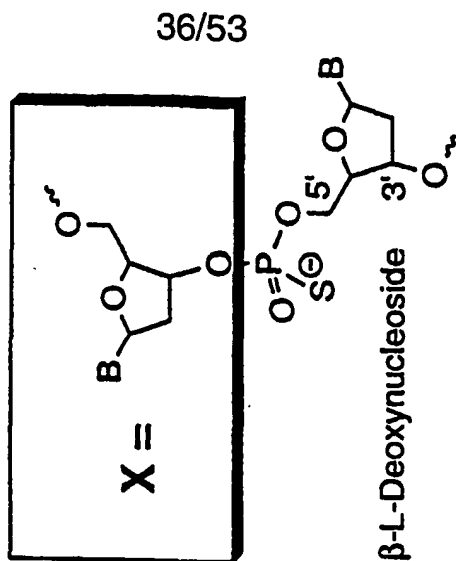


FIG. 18A

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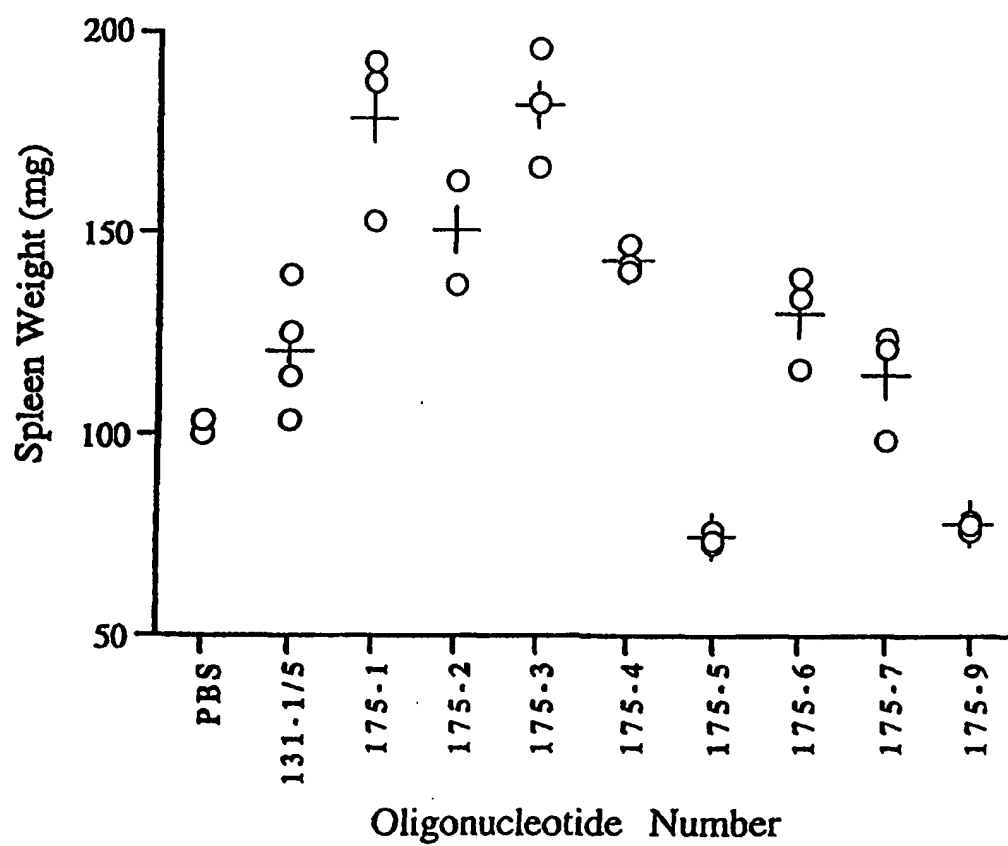


FIG. 18B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

Oligo No.	Sequence & Modification
131-1	5'-CTATCTGACGTTCTCTGT-3'
173-1	5'-CTATXTGACGTTCTCTGT-3'
173-2	5'-CTATCTGACGTTCTXTGT-3'

$X =$ $\sim\sim\sim$ $\left[\text{ribose-2'-O-propargyl} \right]_n$

2'-O-Propargyl-ribonucleoside

FIG. 19A

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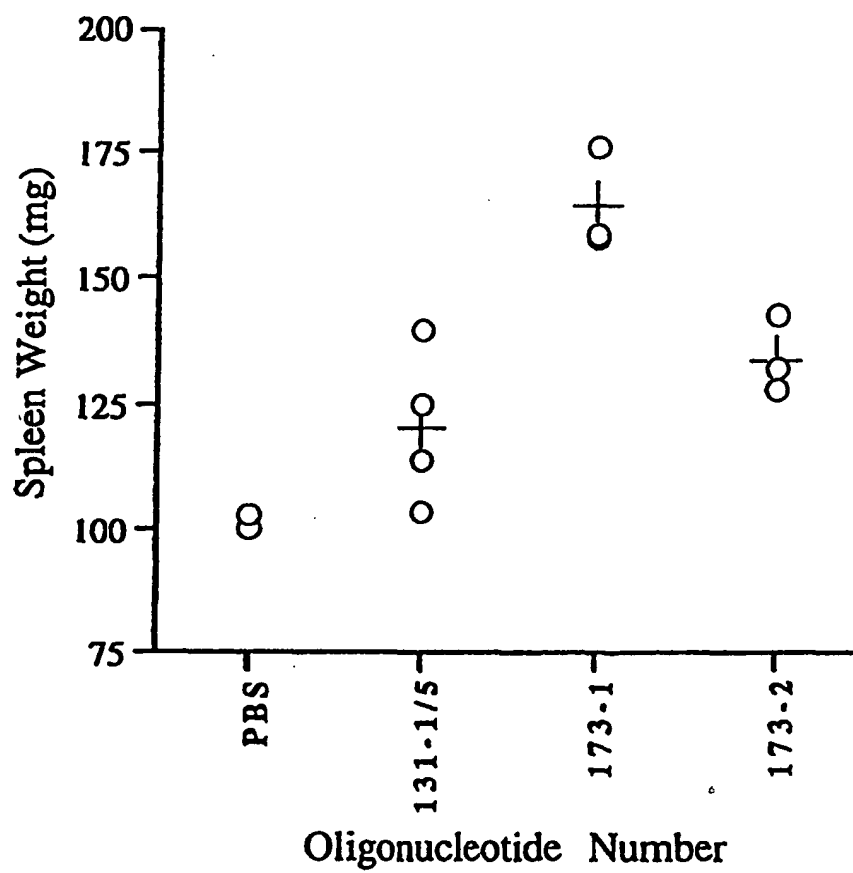


FIG. 19B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND MODIFICATION OF LINKAGES

<i>Oligo No.</i>	<i>Sequence & Modification</i>
133-1	5'-CCTACTAGCGTTCTCATC-3'
143-1	5'-CCTXXTAGCGTTCTCATC-3'
143-2	5'-CCTYYTAGCGTTCTCATC-3'
143-3	5'-CCTZZTAGCGTTCTCATC-3'
143-4	5'-CCTXXTAGCGTVCTCATC-3'
143-5	5'-CCTACTAGG <u>CT</u> CTCATC-3'

X = 1',2'-Dideoxyribose

Y = C3-Linker

Z = 3'-OMe

V = 2'-OMe

FIG. 20A

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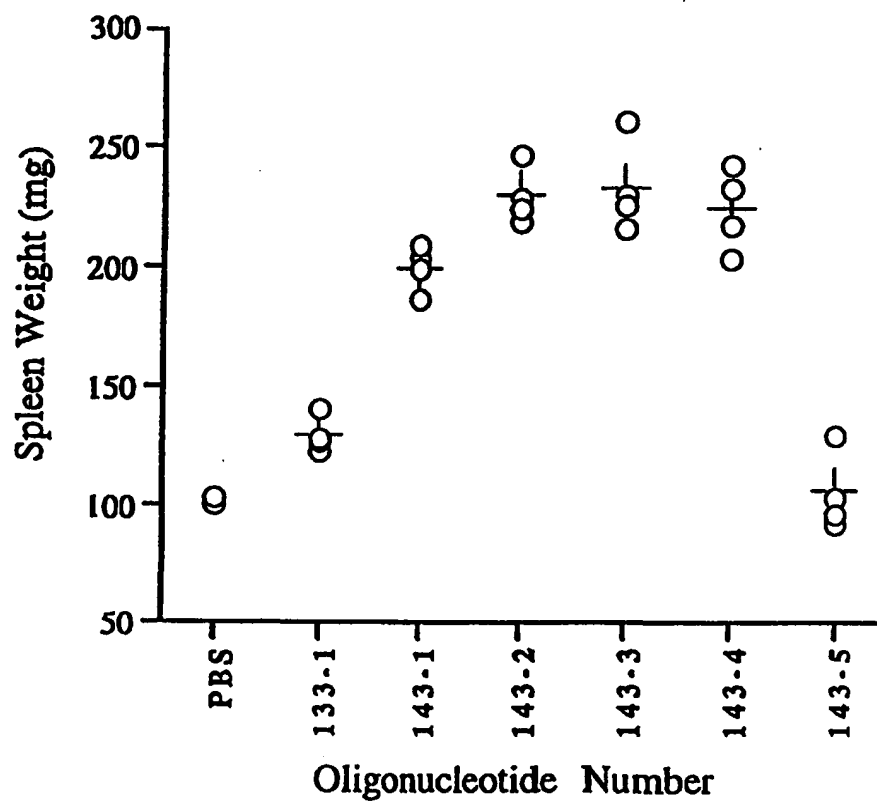


FIG. 20B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND
MODIFICATION OF LINKAGES

Oligo No.	Sequence & Modification
1	5'-CTATCTGACGTTCTCTGT-3' (131-1)
2	5'-CTATCTGACG*TTCTCTGT-3' (E 633L)
3	5'-CTATCTGAG*CTTCTCTGT-3' (E 636)
4	5'-TCTCCCAGCGTGCGCCAT-3'
5	5'-TCTCCCAGCG*TGCG*CCAT-3' (E 603)
6	5'-CTATXTGACG*TTCTCTGT-3' (E 639L)

FIG. 21A

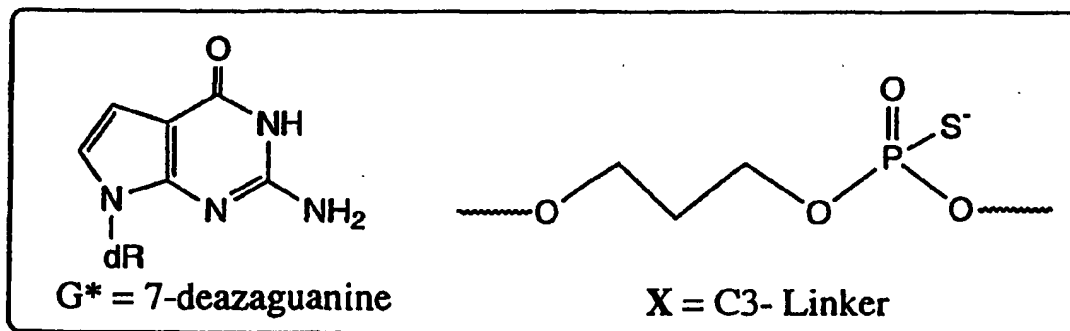


FIG. 21B

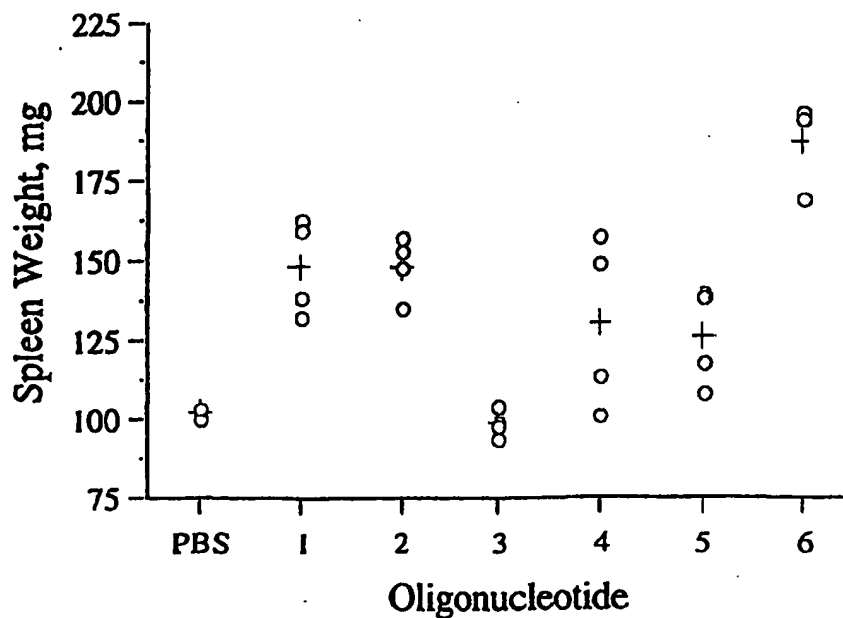
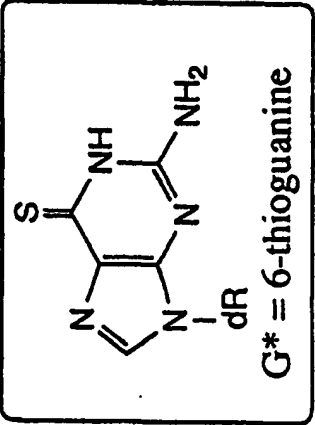


FIG. 21C

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGACG <u>TTCTCTGT</u> -3' (131-1)
2	5'-CTATCTGACG <u>TTCTCTGT</u> -3' (E 682)
3	5'-CTATCTGAG <u>CTTCTCTGT</u> -3' (E 683)



G* = 6-thioguanine

FIG. 22A

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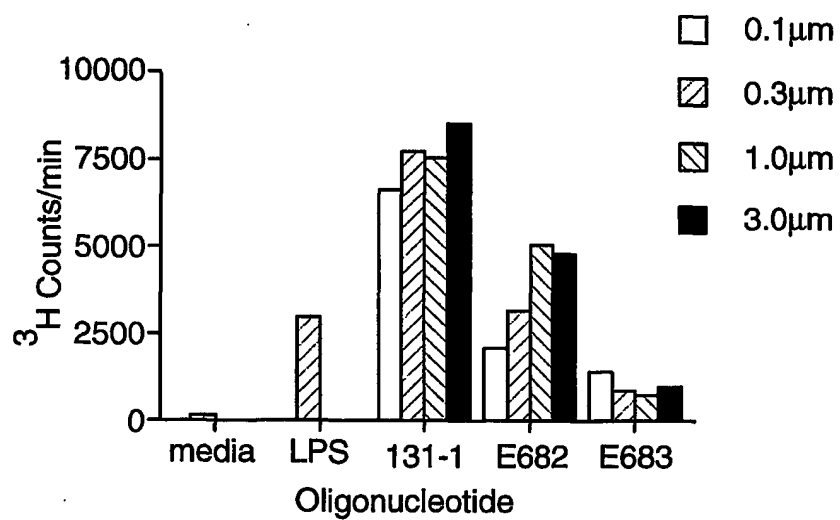


FIG. 22B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

Oligo No.	Sequence & Modification
1	5'-CTATCTGAC <u>CG</u> TTCTCTGT-3'
2	5'-CTATCTGAC* <u>G</u> TTCTCTGT-3'
3	5'-CTATCTGAC <u>CC</u> *TTCTCTGT-3'
4	5'-CTATCTGAC** <u>G</u> TTCTCTGT-3'
5	5'-CTATCTGAC <u>CC</u> **TTCTCTGT-3'

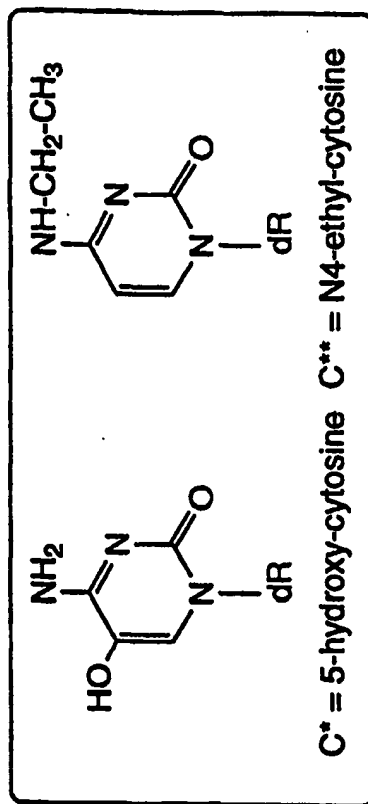


FIG. 23A

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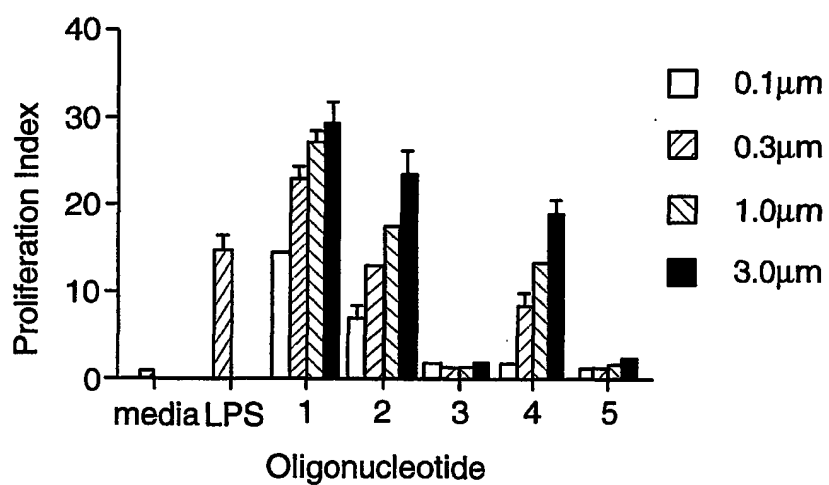
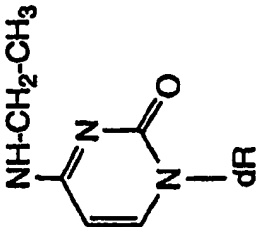
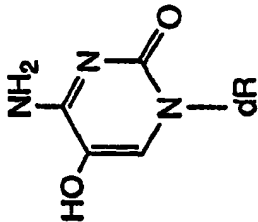


FIG. 23B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGAC <u>GT</u> TTCTCTGT-3'
2	5'-CTATCTGAC* <u>GT</u> TTCTCTGT-3'
3	5'-CTATCTGAC <u>CC</u> *TTCTCTGT-3'
4	5'-CTATCTGAC** <u>GT</u> TTCTCTGT-3'
5	5'-CTATCTGAC <u>CC</u> **TTCTCTGT-3'



C* = 5-hydroxy-cytosine C** = N4-ethyl-cytosine

FIG. 24A

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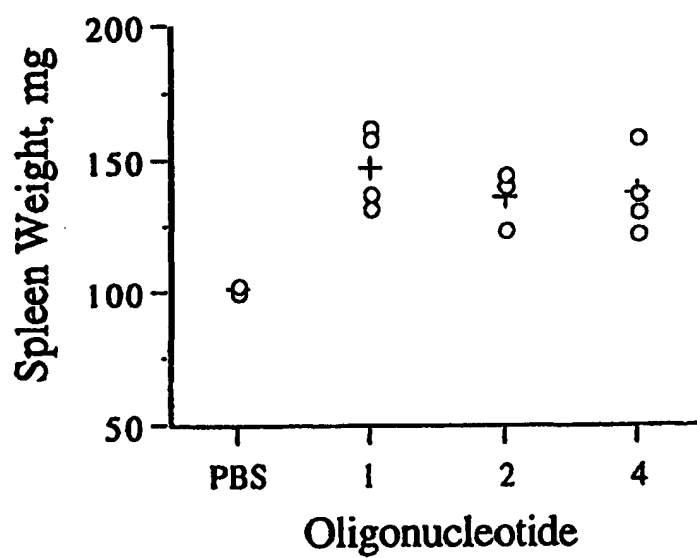


FIG. 24B

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGAC <u>CG</u> TTCTCTGT-3'
2	5'-CTATCTGAC* <u>GT</u> TCCTGT-3'
3	5'-CTATCTGAC <u>CC</u> *TTCTCTGT-3'

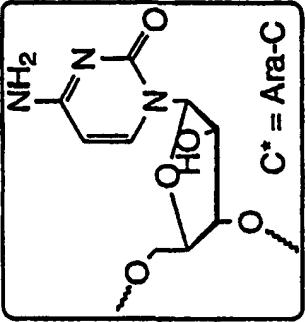


FIG. 25A

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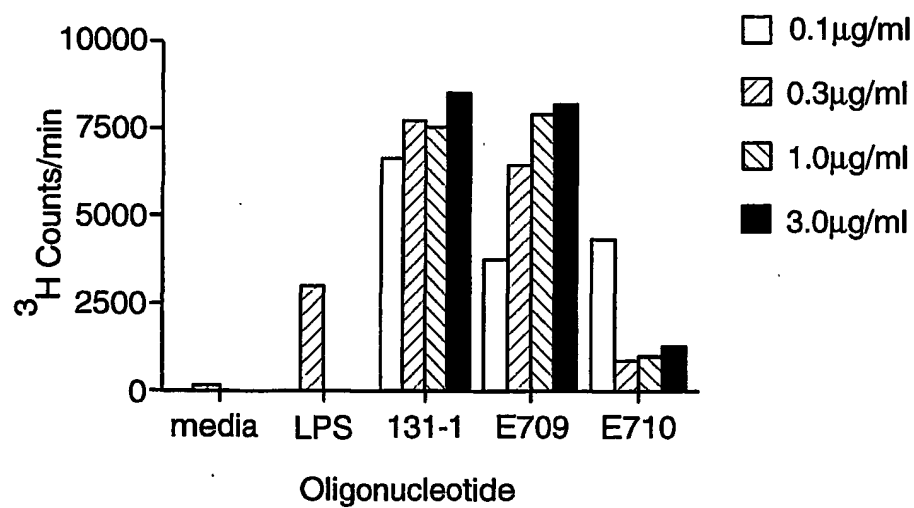


FIG. 25B

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OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES AND SITE OF MODIFICATION

<i>Oligo No.</i>	<i>Sequence & Modification</i>
1	5'-CTATCTGAC <u>CGTTCTCTGT</u> -3' (131-1)
2	5'-CTATCTGAC* <u>GTTCTCTGT</u> -3' (E 647)
3	5'-CTATXTGAC* <u>GTTCTCTGT</u> -3' (E 653)

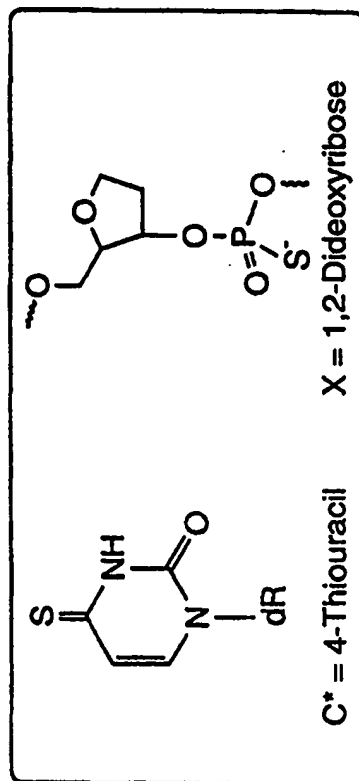


FIG. 26A

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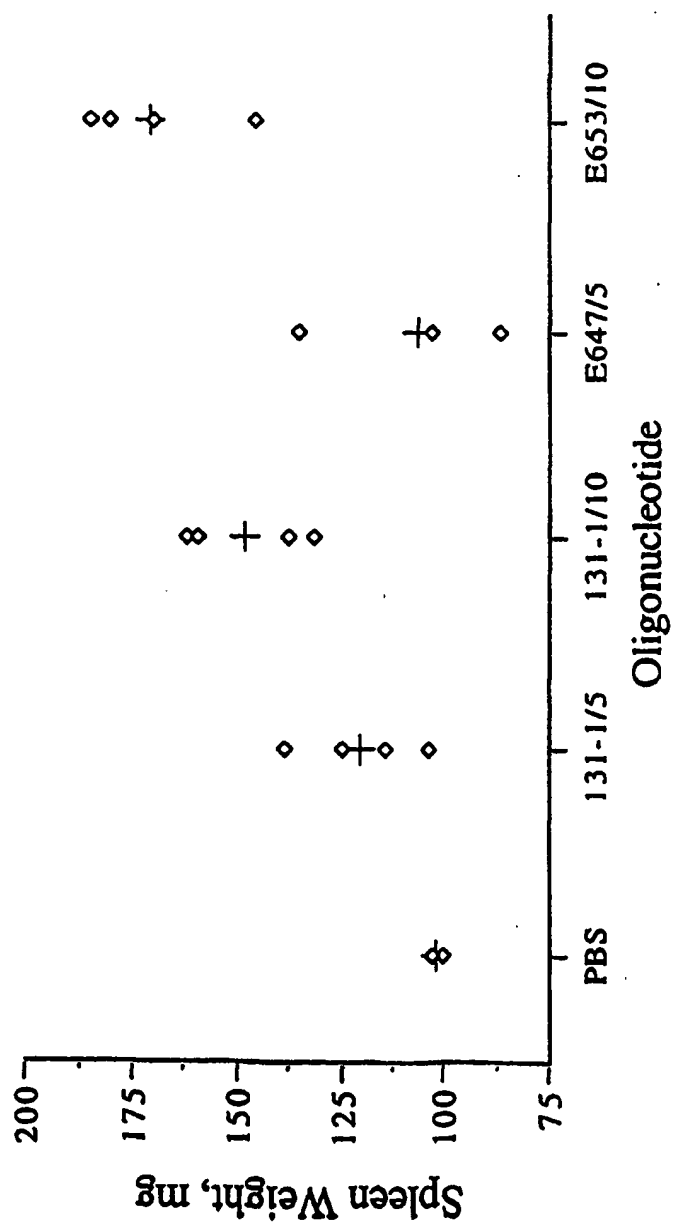
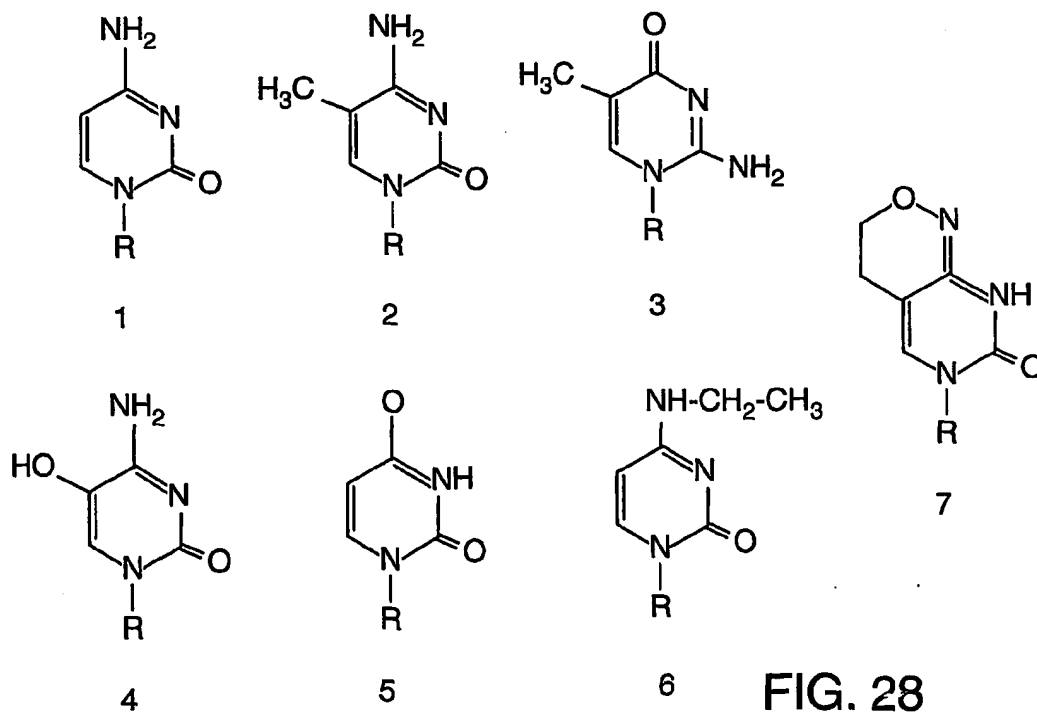
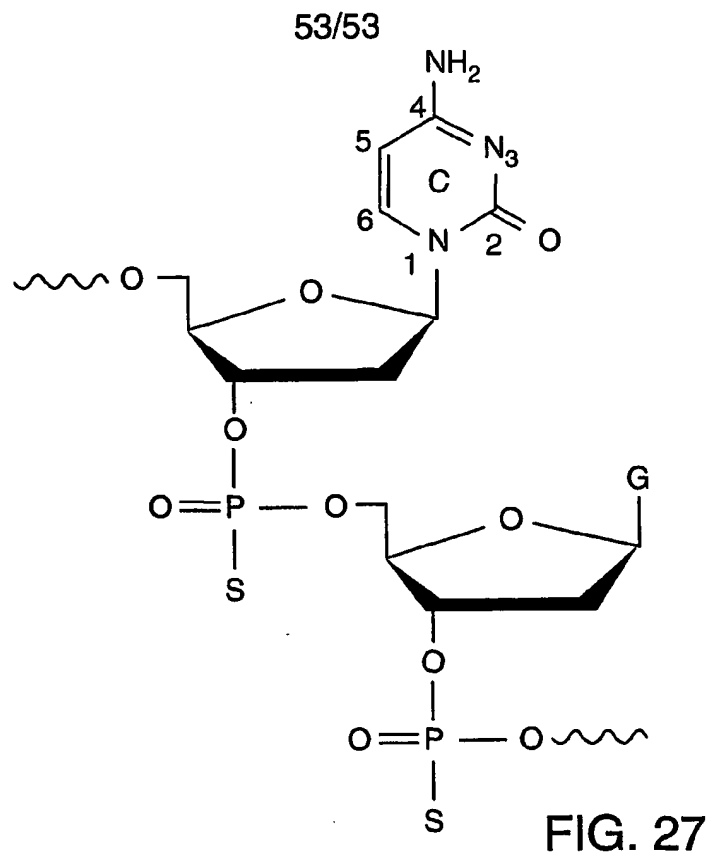


FIG. 26B



(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 April 2002 (04.04.2002)

PCT

(10) International Publication Number
WO 02/026757 A3

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- (21) International Application Number: PCT/US01/30137
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09/712,898 15 November 2000 (15.11.2000) US
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- (74) Agent: **WAYNE A. Keown, Ph.D.**; Law Offices of A. Keown, 500 West Cummings Park, Suite 2900, Woburn, MA 01801 USA (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
- (88) Date of publication of the international search report:
3 January 2003
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 02/026757 A3

(54) Title: MODULATION OF IMMUNOSTIMULATORY ACTIVITY OF IMMUNOSTIMULATORY OLIGONUCLEOTIDE ANALOGS BY POSITIONAL CHEMICAL CHANGES

(57) Abstract: The invention relates to the therapeutic use of oligonucleotides or oligonucleotide analogs as immunostimulatory agents in immunotherapy applications. The invention provides methods for enhancing the immune response caused by immunostimulatory oligonucleotide compounds.

INTERNATIONAL SEARCH REPORT

Initial Application No

PCT/US 01/30137

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C07H21/00 A61K31/70 A61P37/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C07H A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO Q ET AL: "Immunostimulatory activity of CpG containing phosphorothioate oligodeoxynucleotide is modulated by modification of a single deoxynucleoside" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD, GB, vol. 10, no. 10, May 2000 (2000-05), pages 1051-1054, XP004204603 ISSN: 0960-894X	9,24,26, 27,30-38
Y	cited in the application the whole document, in particular Table 1 and page 1053, 2. column, last paragraph - page 1054, 1. column, line 6 --- -/--	1-4,7, 9-38

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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T later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the International search

30 August 2002

Date of mailing of the International search report

17/09/2002

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INTERNATIONAL SEARCH REPORT

In International Application No
PCT/US 01/30137

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO Q ET AL: "Site of Chemical Modifications in CpG Containing Phosphorothioate Oligodeoxynucleotide Modulates its Immunostimulatory Activity" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD, GB, vol. 9, no. 24, 20 December 1999 (1999-12-20), pages 3453-3458, XP004185533 ISSN: 0960-894X cited in the application	9,24,26, 27,30-38
Y	the whole document, in particular: Table 1, and page 3457, 5. paragraph	1-4,7, 9-38
X	AGRAWAL S ET AL: "ANTISENSE THERAPEUTICS" CURRENT OPINION IN CHEMICAL BIOLOGY, CURRENT BIOLOGY LTD, LONDON, GB, vol. 2, no. 4, 1998, pages 519-528, XP000979488 ISSN: 1367-5931	1-4,7, 12,23, 24,26, 27,30-38
Y	page 523, 1. column, last paragraph; page 524, Figure 4	1-4,7, 9-38
X	CHAIX, C. ET AL.: "3'-3'-LINKED OLIGONUCLEOTIDES: SYNTHESIS AND STABILITY STUDY" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, vol. 6, no. 7, 1996, pages 827-832, XP002211794	25-27, 30-38
Y	page 827, last 8 lines and page 828, 2. paragraph, first two lines and Table 1	1-4,7, 9-38
Y	KLINMAN D M: "Therapeutic applications of CpG -containing oligodeoxynucleotides" ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT, MARY ANN LIEBERT, INC., NEW YORK, US, vol. 8, 1998, pages 181-184, XP002128519 ISSN: 1087-2906 the whole document	1-4,7, 9-38
P,X	YU D ET AL: "Accessible 5'-end of CpG-containing Phosphorothioate Oligodeoxynucleotides is essential for immunostimulatory activity" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD, GB, vol. 10, no. 23, 4 December 2000 (2000-12-04), pages 2585-2588, XP004219767 ISSN: 0960-894X the whole document	20,21, 25-38

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INTERNATIONAL SEARCH REPORT

In ☐ International Application No
PCT/US 01/30137

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>KANDIMALLA EKAMBAR R ET AL: "Effect of chemical modifications of cytosine and guanine in a CpG-motif of oligonucleotides: structure-immunostimulatory activity relationships" BIOORGANIC & MEDICINAL CHEMISTRY, ELSEVIER SCIENCE LTD, GB, vol. 9, March 2001 (2001-03), pages 807-813, XP002198015 ISSN: 0968-0896 Figure 1</p> <p>-----</p>	<p>1-6, 12, 23, 26, 30-38</p>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/30137

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 26-38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.